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**Purinergic signalling in  
malignant melanoma**

**Nicholas White**

*A thesis submitted for the degree of Doctor of Medicine  
at the University of London*

**2005**

**Royal Free and University College Medical School  
University College London**



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## Abstract

Malignant melanoma is an aggressive cancer that originates from melanocytes, the pigment producing cells of the skin. The incidence of melanoma is increasing and the outcome for patients with advanced disease remains poor. New therapies for melanoma are urgently needed as no current systemic treatment is effective.

Interactions between the nervous system and epidermal melanocytes have been suspected on the basis of their common embryological origin from the neural crest. Both melanocytes and melanomas are innervated by autonomic nerves with acetylcholine and noradrenaline acting as transmitter molecules. It is now well established that the purine nucleotide adenosine triphosphate (ATP) is a co-transmitter with noradrenaline in sympathetic nerves and with acetylcholine in parasympathetic nerves. ATP acts on extracellular receptors which have been characterised to consist of a number of subtypes. ATP acting on these receptors is involved with both rapid signalling in neurotransmission and also long term signalling in cell proliferation, differentiation and apoptosis.

This thesis demonstrates the presence of functional purinergic receptors at tissue, cell and molecular level in both excised specimens of melanomas and melanoma cell lines. Activation of extracellular ATP receptors shows statistically-significant, dose-dependent changes in melanoma cell number which are reversible by selective antagonists. Proliferation of melanoma cells is mediated through the P2Y<sub>2</sub> metabotropic receptor subtype. An anti-proliferative effect of ATP receptors is mediated through the P2Y<sub>1</sub> metabotropic receptor subtype and melanoma apoptosis is mediated through the P2X<sub>7</sub> ionotropic receptor subtype. Treatment of an *in vivo* model of melanoma with ATP causes a significant reduction in the rate of growth of the cancer and prevents systemic weight loss. In addition there is a progressive loss of

P2X<sub>3</sub> receptor expression in malignant melanomas with a poor prognosis compared to those with a good prognosis.

This is the first study to demonstrate the expression of functional P2 receptors in melanomas. Purinoreceptors in melanoma may be both a therapeutic target and a diagnostic marker in the management of this disease.

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## Abbreviations

ABC, ATP-binding cassette

ADP, adenosine 5'-diphosphate

ATP, adenosine 5'-triphosphate

BzATP, 2'-3'-O-(4-benzoyl-benzoyl) adenosine 5'-triphosphate

CaMKII, calmodulin-dependent protein kinase II

CRGP, calcitonin gene-related peptide

CFTR, cystic fibrosis conductance regulator

DPBS, Dulbeccos phosphate buffered saline

FLIPR, Fluorometric Imaging Plate Reader

GAGs, glycosaminoglycans

IGF-1, insulin like growth factor 1

ILP, isolated limb perfusion

IP3, inositol 1,4,5-triphosphate

KN-62, 1-N,O-bis-[5-isoquinoline-sulfonyl]-N-methyl-L-tyrosyl)-4-phenyl-piperazine

2MeSADP, 2-methylthioadenosine-5-diphosphate

MSH, Melanocyte stimulating hormone

MRS 2179, N<sup>6</sup>-methyl-2'-deoxyadenosine 3',5'-bisphosphate

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NF-κB nuclear factor-κB

NGF, nerve growth factor

PBS, phosphate buffered saline

PLC, phospholipase C

PPADS, Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid

POMC, proopiomelanocortin

RT-PCR, reverse transcriptase polymerase chain reaction

TUNEL, terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate  
nick end-labelling

TGF $\alpha$ , transforming growth factor alpha

UV, ultraviolet

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## Preface

In this preface, I give a short outline of the different chapters in this thesis. The work presented here focuses on the expression and role of purinergic signalling in melanoma. The use of purinergic receptors in clinical practice as a target for treatment or a marker of malignant melanoma is considered.

Chapter 1 provides a general introduction to malignant melanoma and purinergic signalling. The rapidly expanding field of purinoreceptors is discussed including the history, classification and characterisation of the different receptor subtypes. The existing evidence for the presence of purinergic receptors in melanoma is discussed.

In the first experimental chapter (Chapter 2), the functional expression of P2Y receptors is studied. Metabotropic purinoreceptors are characterised in melanoma tissue and cells and P2Y receptors are shown to regulate melanoma cell proliferation with activation of the P2Y<sub>1</sub> receptor subtype causing a decrease in cell number and activation of the P2Y<sub>2</sub> receptor subtype causing an increase in cell number.

Chapter 3 explores the role of ionotropic P2X receptors in the regulation of cell growth. Using a variety of techniques the presence of functional P2X<sub>7</sub> receptors is demonstrated. Activation of this receptor is shown to cause apoptosis leading to a decrease in cell number.

The next chapter (Chapter 4) explores whether purinergic signalling is a potential therapeutic target for the treatment of melanoma *in vivo*. The nude (athymic) mouse inoculated with A375 human melanoma cells is a widely studied model of malignant melanoma. Treatment of this model with ATP caused a reduction in the rate of growth of melanoma.

The value of purinoreceptors as diagnostic markers is examined in the final experimental chapter (Chapter 5). Using immunohistochemistry, a large number of both benign and malignant pigmented lesions of different histological subtype are examined. There is a significant loss of expression of the P2X<sub>3</sub> receptor in melanomas when compared to benign naevi.

In Chapter 6, the major findings of this thesis are summarised and explored. The relevance of these findings to clinical practice are then discussed. The future direction of research in this field is also suggested.



## **Chapter One**

### **Introduction**

## **Part One: Malignant melanoma**

Cutaneous malignant melanoma is a skin cancer derived from melanocytes, the pigment-producing cells of the skin. The incidence of melanoma is increasing and the outcome for patients with advanced disease remains poor as there is resistance to current methods of treatment. Therefore, new treatment strategies are urgently needed.

### **1.1.1. The skin**

The skin is a complex organ covering the surface of the body constituting 15% of its total weight. It is the largest organ of the body and performs many vital homeostatic functions. These are physical protection, protection against ultraviolet light, protection against microbial invasion, prevention of fluid loss, regulation of body temperature, sensation and immunological surveillance (Kanitakis 2002). The skin is anatomically divided into three distinct layers comprising different cell types (Fig. 1.1). The most superficial layer is the epidermis, the middle layer the dermis and the deep layer is subcutaneous fat (Murphy 1997).

The epidermis is composed of stratified squamous epithelium, consisting of cells called keratinocytes, which are derived from ectoderm. The keratinocytes provide the barrier function of the skin. Epidermal cells undergo keratinisation in which their cytoplasm is replaced with keratin as the cells dies and becomes more superficial. The epidermis is composed of the following five layers (Fig. 1.2A), from deep to superficial:

Figure 1.1 Cross section of normal skin

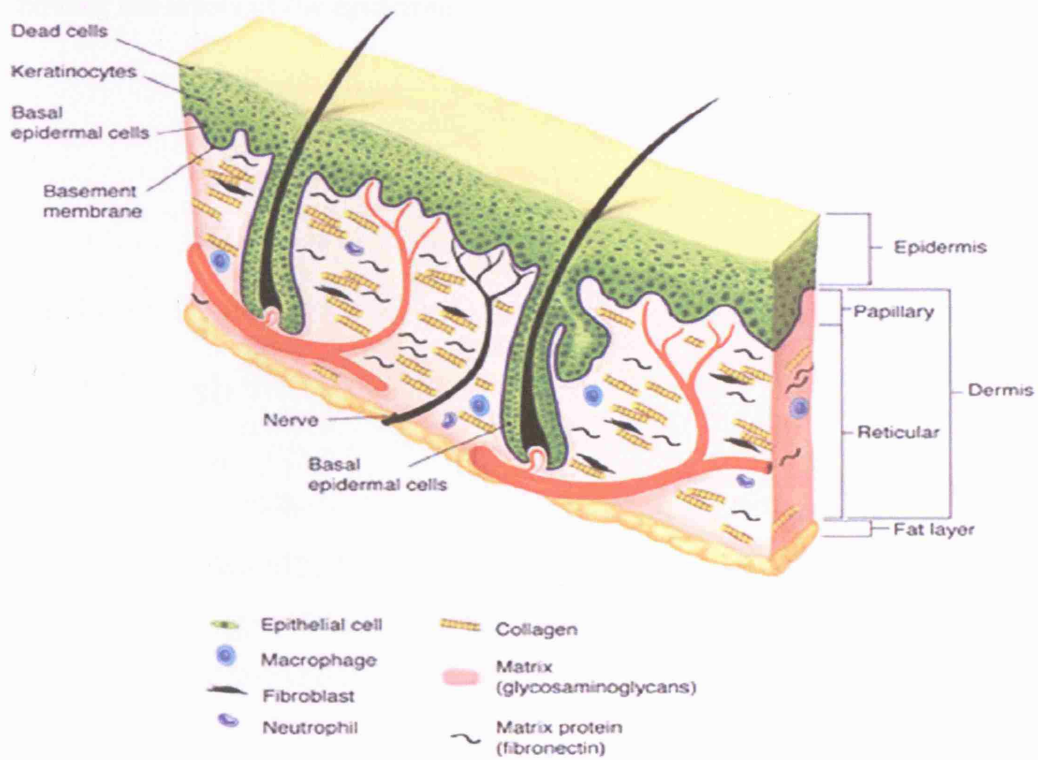
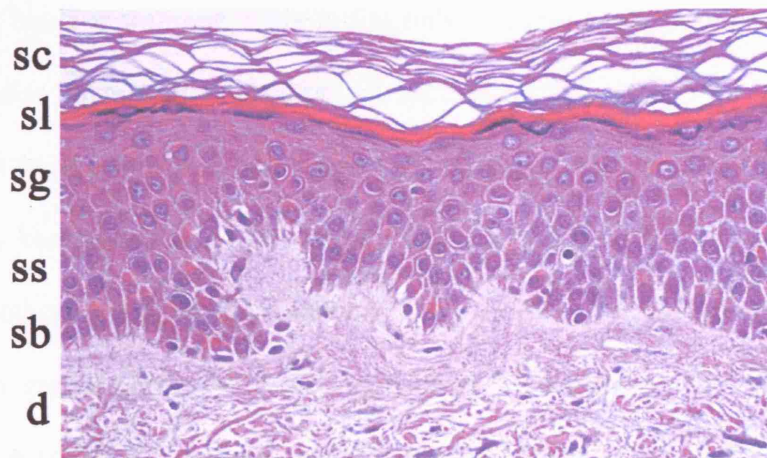


Figure 1.2A Haematoxylin and eosin staining of a cross-section of normal human skin showing the layers of the epidermis.



**sc-stratum corneum**

**sl-stratum lucidum**

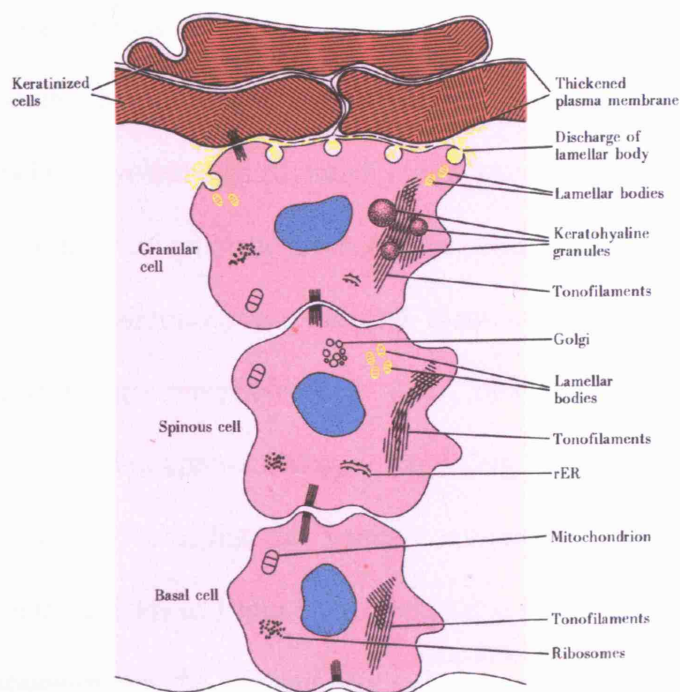
**sg-stratum granulosum**

**ss-stratum spinosum**

**sb-stratum basale**

**d-dermis**

Figure 1.2B Keratinocytes in the epidermis



Stratum basale; this is also known as the basal layer. This is a single layer deep and the cells within this layer have cytoplasmic projections, which link them firmly to the underlying basal membrane. This is the only actively proliferating layer of the epidermis and also contains melanocytes (see section 1.1.2.).

Stratum spinosum; this is also known as the spiny layer. This consists of 5-15 layers of large keratinocytes which produce keratin, the cells within this layer are joined to each other by tonofibrils (spines).

Stratum granulosum; the stratum granulosum contains mature keratinocytes which possess cytoplasmic granules of keratohyalin. This layer is sometimes called the granular layer because of these granules. The stratum granulosum is the predominant site of protein synthesis and consists of approximately 3 layers of keratinocytes.

Stratum lucidum; this layer is only present in the thick skin of the palms and soles of the feet and is a clear layer.

Stratum corneum; this outermost layer contains non-viable keratinized cells. The thick cells of this layer protect against trauma, fluid loss and bacterial invasion

Epidermal keratinocytes originate from mitotic divisions of stem cells of the basal layer; the daughter keratinocytes produced there migrate towards the skin surface while undergoing morphological and biochemical differentiation (keratinisation), and are shed in approximately 30 days (Fig 1.2B). The epidermis has an average thickness of 100  $\mu\text{m}$ , but this varies considerably with the body area considered (50  $\mu\text{m}$  on the eyelids to 1 mm on the palms and soles).

While keratinocytes are the predominant cell type of the epidermis, it does contain other cells. Langerhans cells form part of the immune system and function as

antigen presenting cells. Merkel cells are mechanoreceptors and form part of the sensory nervous system. Melanocytes are also present in the stratum basale.

The intermediate layer is the dermis which accounts for 95% of the thickness of the skin and is of mesodermal origin. It is divided into a papillary and reticular dermis. The papillary dermis is superficial and contains more cells and finer collagen fibres, while the reticular dermis is deeper and contains fewer cells and coarser collagen fibres. The dermis is composed of the following:

Collagen fibres; these fibres are produced by fibroblasts and are responsible for much of the strength of the skin. Different subtypes of collagen are present and the normal ratio of type 1 to type 3 collagen is 5:1.

Elastin (fibronectin) fibres; these are secreted by fibroblasts and are responsible for the elastic recoil of the skin.

Ground substance; this consists of glycosaminoglycans (GAGs), hyaluronic acid, dermatansulfate and chondroitin sulphate. GAGs are secreted by fibroblasts and ground substance forms the matrix around the collagen and elastin fibres.

The deep layer of the skin consists of subcutaneous fat. The skin also contains vessels and nerves; as well as appendages such as hair follicles and eccrine, apocrine and sebaceous glands.

### **1.1.2. Melanocytes**

Melanocytes originate from the neural crest and migrate into the epidermis where they produce melanin, the main natural pigment of the skin. They are distributed regularly among basal keratinocytes, at a ratio of 1 melanocyte for 4-10 basal keratinocytes (Fig 1.3A). Their density reaches 500-2,000 cells per mm<sup>2</sup> of cutaneous surface, with regional variations (maximal density on genital skin).



Figure 1.3A Melanocytes in the epidermis

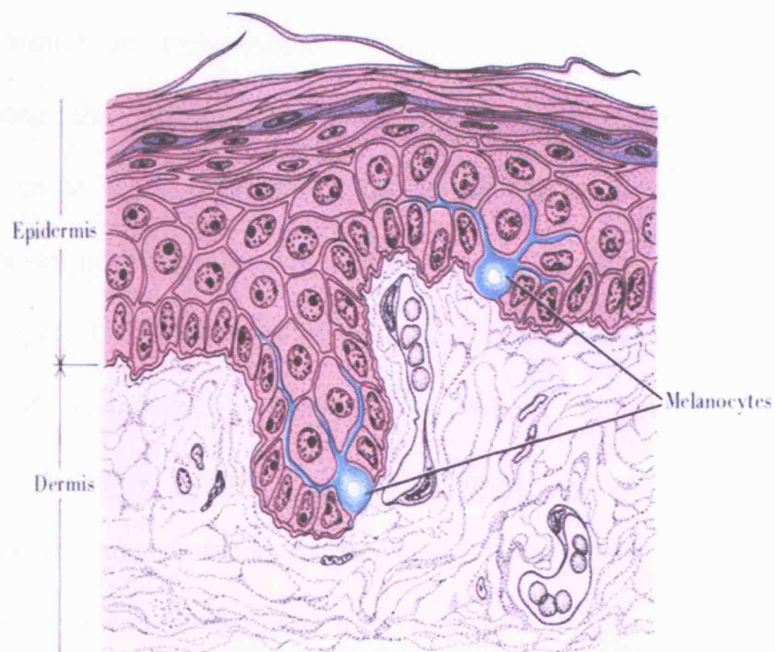
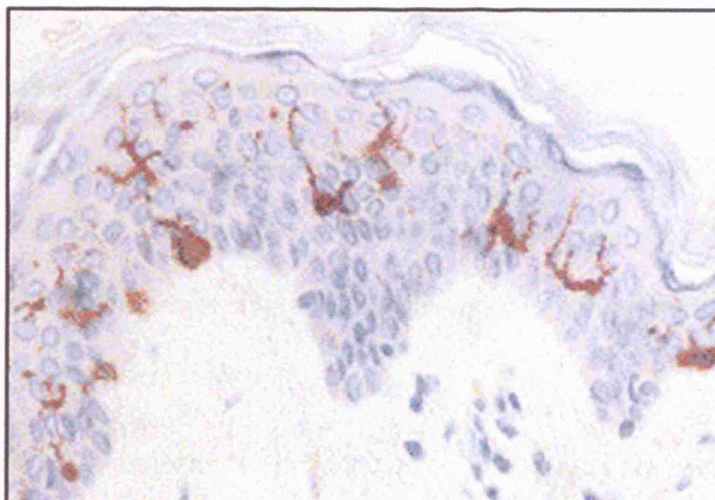


Figure 1.3B Staining of epidermal melanocytes with S100



Melanin is produced through the enzymatic activity of tyrosinase from the substrate tyrosine and stored in melanosomes. Mature melanosomes are subsequently transported along the dendritic processes of melanocyte and are eventually transferred, by an as yet poorly-understood mechanism, to adjacent keratinocytes where they form an umbrella-like cap over the nucleus, protecting it from injurious effects of UV light. The "epidermal melanin unit" consists of one melanocyte and approximately 36 associated keratinocytes to which the melanocyte delivers melanosomes (Fitzpatrick and Breathnach 1963). The ethnic variations in pigmentation are due to differences in activity of melanocytes and distribution of melanosomes within the epidermis, and not to a different number of melanocytes. In routinely stained skin sections (haematoxylin-eosin), melanocytes appear as basal cells with a basophilic nucleus and a clear cytoplasm. Ultrastructurally, they have an electron lucent cytoplasm containing loose intermediate filaments and melanosomes at various maturation stages. Melanocytes can be recognised by histochemical stains that reveal melanin (such as the ammoniacal silver nitrate reaction of Fontana-Masson), or by the DOPA histoenzymatic reaction. They can also be confidently identified with antibodies recognising melanocyte-specific antigens such as the S100 protein (Fig. 1.3B) (Kanitakis 1998).

In addition to cutaneous melanocytes, there are also extracutaneous melanocytes found in the human body. These are at a number of sites and include the retina of the eye, the leptomeninges of the brain stem and ganglion of the sympathetic, parasympathetic and enteric nervous system (Boissy 1998). These autonomic ganglia and nerves are the other tissues derived from the neural crest along with melanocytes.

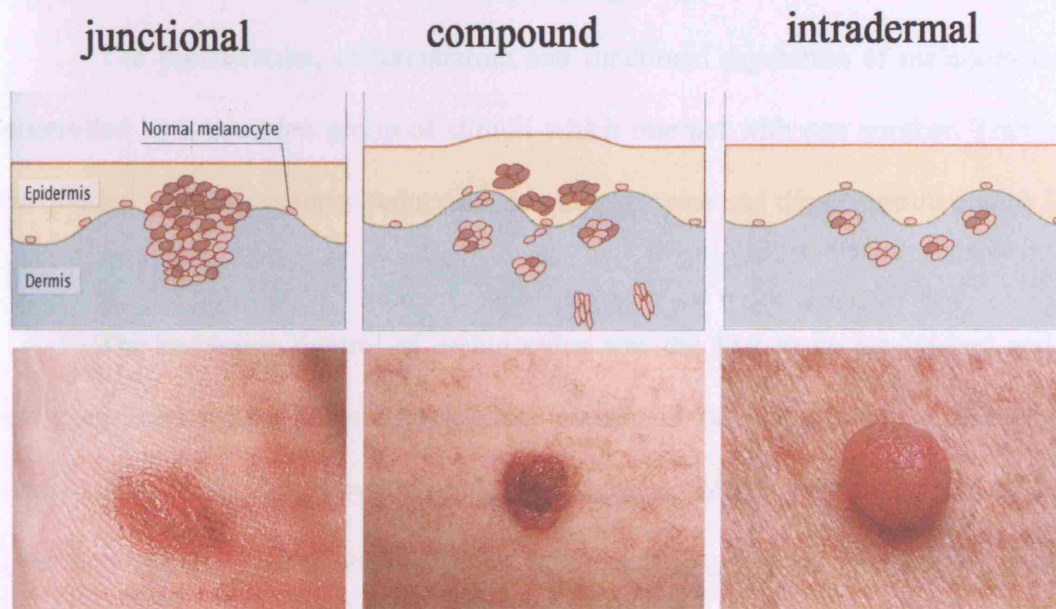
Melanocytic naevi (moles) are localized collections of melanocytes. Most people have up to ten. The clinical and histological features are shown in figure 1.4.

Figure 1.4 Benign melanocytic naevi

Benign melanocytic naevi are common skin lesions that arise from melanocytes.

Figure 1.4

### 1.1.3 Control and regulation of melanocyte growth



Benign melanocytic naevi are common skin lesions that arise from melanocytes. They are characterized by a proliferation of melanocytes in the epidermis, dermis, or both.

The junctional naevus is characterized by a large cluster of melanocytes at the epidermal-dermal junction. The compound naevus is characterized by melanocytes at the junction and some in the dermis. The intradermal naevus is characterized by melanocytes only in the dermis.

The clinical photographs show the appearance of these naevi on the skin. The junctional naevus is a flat, brownish lesion. The compound naevus is a slightly raised, dark brown lesion. The intradermal naevus is a raised, reddish-brown lesion.

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They begin to appear in childhood and may later progress from junctional to intradermal and compound moles during life.

### **1.1.3. Control and regulation of melanocyte growth**

The proliferation, differentiation and functional regulation of melanocytes is controlled by a complex group of stimuli which interact with one another. They can be divided into four groups: endocrine, neural, paracrine and direct stimulation by UV light (Fig. 1.5).

The endocrine control of melanocytes was the first to be established and as early as 1912 it was known that factors present in bovine pituitary extract could darken frog skin. Melanocyte stimulating hormone (MSH) was described in 1954 (Lerner et al. 1954) and since then the melanocortins, a group of proteins derived from the precursor protein proopiomelanocortin (POMC), have been characterised. Differential processing of POMC, a molecule now known to be produced not only in the pituitary but also in other tissues including the skin, gives rise to several peptides including  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH, ACTH,  $\gamma$ -lipotropin and  $\beta$ -endorphin. Melanocortins bind their specific receptors which are a family of G-protein-coupled seven-membrane spanning molecules linked to activating adenylate cyclase. Sex hormones (androgens and oestrogens) have been implicated in changes in skin colour and glucocorticoids also have an effect on melanocyte function.

Melanocytes are in an environment where they are attached to a basement membrane with a rich vascular plexus and surrounded by keratinocytes; which regulate melanocytes in a paracrine manner. Keratinocytes have been shown to release locally active growth factors such as insulin like growth factor 1 (IGF-1) and transforming growth factor alpha (TGF $\alpha$ ) in response to UV stimulation. Furthermore

Figure 1.5 Multifactorial regulation of melanocyte function

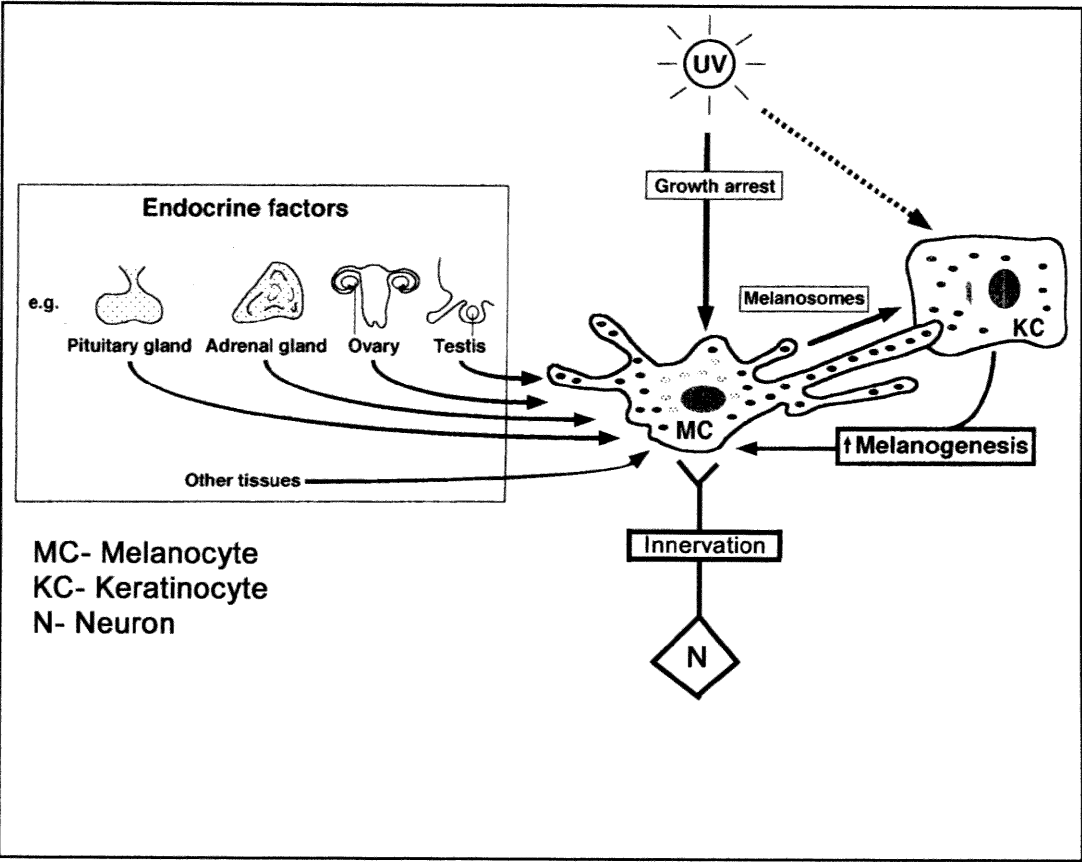


Figure modified from The Pigmentary System; Norlund 1998.

melanocytes are known to express functional receptors for these growth factors (Halaban 1991;Norris et al. 1994;Halaban et al. 1988).

One of the most obvious responses to sun exposure is increased skin darkening (i.e., tanning). This is mainly mediated by direct action of UV light on melanocytes. It has been clearly shown that ultraviolet irradiation of cultured melanocytes causes a dose-dependent decrease in proliferation and an increase in melanin production (melanogenesis) (Friedmann and Gilchrest 1987;Libow et al. 1988).

Interactions between the nervous system and epidermal melanocytes have been suspected on the basis of their common embryological origin from the neural crest but little published work exists to support this. It has been suggested that melanocytes are innervated by autonomic nerves (Hara et al. 1996c) with both acetylcholine and noradrenaline acting as transmitter molecules (Hu et al. 2000c). There are no previous reports of the sympathetic cotransmitter ATP (Burnstock 1990) acting on melanocytes. As well as short-term rapid signalling, these nerves may also release other transmitter molecules that have long term trophic actions such as the neuropeptides nerve growth factor (NGF) and calcitonin gene-related peptide (CRGP) (Hara et al. 1996;Yaar et al. 1994).

#### **1.1.4. Epidemiology and aetiology of melanoma**

Malignant melanoma, a cancer first described in 1787 by John Hunter (Bodenham 1968), has an incidence in the UK of 8 per 100,000 of the population per year and this figure is steadily increasing with a 24% increase in the last 5 years (Quinn et al. 2004). The worldwide incidence varies considerably, with the highest risk being in Queensland, Australia, where it is 33 per 100,000 per year. The incidence in black populations is lowest at 0.6 per 100,000 per year.



The development of melanoma has been strongly linked to exposure to ultraviolet (UV) light and epidemiological studies show a complex association of melanoma with sun exposure (Elder 1989). The highest incidences are in fair-skinned people living near the equator and the pattern of sun exposure in either sex is reflected by the site of melanoma development. Nearly three times as many cases occur on the trunk in men as in women (35% and 14%, respectively); a similar ratio, but in reverse, occur on the lower limbs (18% in men and 50% in women) (Quinn et al. 2004). The condition appears to be initiated by an episode of severe sunburn rather than constant exposure, blistering sunburn during childhood and adolescence is associated with an increased incidence of later melanoma (Lew et al. 1983).

Other risk factors include: a previous melanoma, which increases the risk of a second primary three and a half times; more than twenty benign pigmented naevi, which increases the risk three times; and family history in first degree relatives, which increases the risk one and a half times. Xeroderma pigmentosum, a familial condition associated with the failure of DNA transcription, carries a large risk.

#### **1.1.5. Diagnosis and classification of melanoma**

The clinical diagnosis of melanoma is based on the seven-point checklist of major and minor features (MacKie et al. 1994) Major features are: (1) a change in size; (2) an irregular shape; (3) an irregular colour. Minor features are: (4) largest diameter 7 mm or more; (5) inflammation; (6) oozing; (7) change in sensation. Lesions with any of the major features or three minor ones are suspicious of melanoma and should be treated by complete excision biopsy with a 2 mm margin and sent for histopathological examination.

Histologically, a malignant melanoma usually consists of nests and groups of melanocytes which are cytologically malignant with large nuclei (Fig. 1.6A). There is invasion of both the epidermis and dermis and sometimes into the subcutaneous fat. Currently there are sixteen specific sets of data that make up a histological report on a melanoma specimen (Balch et al. 2001a). Of these the four most important are: Breslow thickness, growth phase, ulceration and lymphatic spread (Balch et al. 2001b). In 1970, Breslow described the thickness of the primary tumour measured in millimetres (Fig. 1.6B) and showed a direct relationship to clinical outcome (Breslow 1970a). The next best histological marker of prognosis is radial (horizontal) or vertical growth phase (Murphy et al. 1985). Radial growth indicates the tendency of a melanoma to grow horizontally within the epidermal and superficial dermal layers, often for a prolonged period of time. During this phase of growth melanomas do not have the capacity to metastasize. With time, the pattern of growth changes to include a vertical component and the melanoma now grows downward into the deeper dermal layers. This is represented clinically by the formation of a nodule in a previously flat melanoma and correlates with the emergence of metastatic potential. Ulceration is a manifestation of vertical growth phase; the overlying epidermis is eroded as the melanoma expands through it. An ulcerated melanoma carries a worse prognosis than an unulcerated melanoma. The main route of metastasis for melanomas is lymphatic spread to the regional lymph node basin (neck, axilla or groin). Macroscopic lymphadenopathy is also a poor prognostic sign.

There are various subtypes of melanoma with different characteristics; examples are shown in figure 1.7. Superficial spreading melanoma is the most common subtype (70%) and may develop on any part of the body at any age. These tend to be thin melanomas, the radial growth phase predominates and prognosis is

Figure 1.6A Haematoxylin and eosin staining of a superficial spreading melanoma in vertical growth phase.

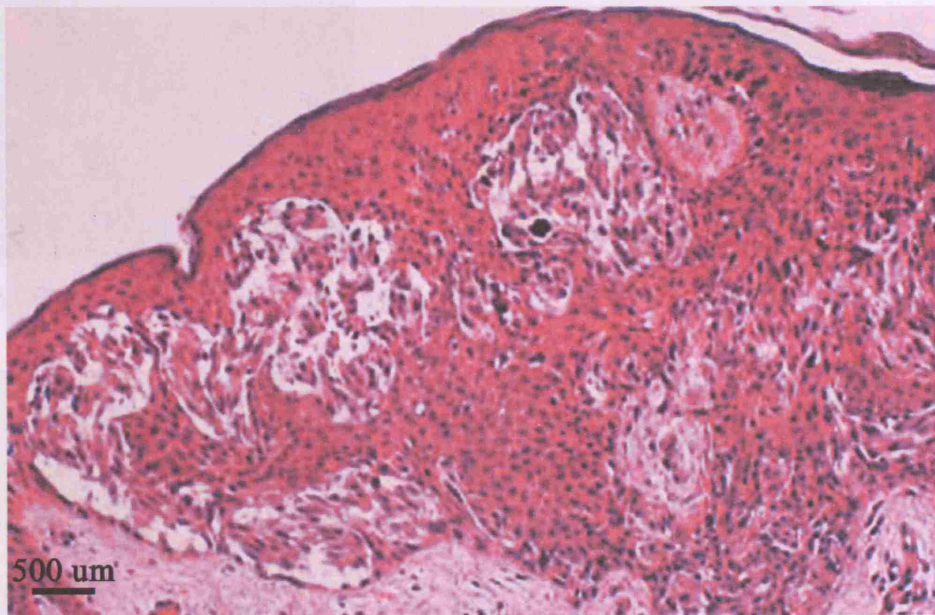


Figure 1.6B Diagrammatic representation of Breslow thickness

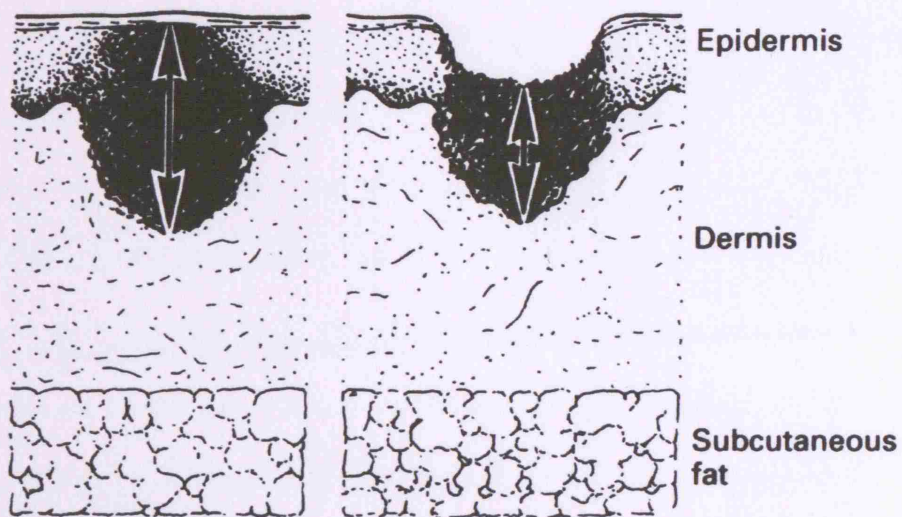


Figure 1.7 Macroscopic subtypes of melanoma



Acral lentiginous



Nodular



Lentigo maligna melanoma



Superficial spreading

usually good. Nodular melanoma accounts for 15-30% of all melanomas, histologically there is no radial growth phase resulting in a raised pigmented lump. This type of melanoma has a significantly worse prognosis. Lentigo maligna melanoma occurs on the face and sun-exposed extremities of the elderly and accounts for approximately 5% of melanomas. It is characterised by the formation of discrete nodules of invasive malignancy (vertical growth phase) in a background of lentigo maligna. Acral lentiginous melanomas develop on palmar, plantar and subungual skin and may be amelanotic. They represent less than 3% of all melanomas but are particularly common in black and asian races. They have a poor prognosis. Desmoplastic melanoma is usually found on the head and neck, commonly recur after excision, are not normally pigmented and grow to a large size. They make up only 1% of melanomas and also carry a poor prognosis.

#### **1.1.6. Treatment and outcome of melanoma**

Surgical excision remains the treatment of choice for melanoma. Diagnostic biopsy, if positive for melanoma, should be followed by definitive excision which should include a margin of normal tissue in proportion to the Breslow thickness (Bishop et al. 2002a); this is to reduce the risk of local recurrence. Where there is spread to the regional lymph node basin, surgical lymphadenectomy should be undertaken. Adjuvant therapies for metastatic melanoma are in constant development as no current option is sufficiently effective. Melanomas are not radio-sensitive, though radiotherapy may provide palliation in cases of nerve compression. Systemic chemotherapy is toxic and provides minimally prolonged survival. Immunotherapies for melanoma include the use of vaccines to raise an anti-melanoma antibody response, monoclonal antibody therapy and systemically delivered cytokines such as

interferons and interleukins. These developing therapies are currently under evaluation.

The overall prognosis for patients with melanomas varies considerably (Balch et al. 2001b). A patient with a thin melanoma (<1 mm) which is completely excised has an 88% survival at 10 years, this reduces to 60% for a melanoma >2 mm and to 30% for a melanoma >4 mm which is ulcerated. For a patient with metastatic spread to the regional lymph node basin survival is at best 60% at 5 years post surgical lymphadenectomy. Any patient with distant solid organ metastasis is rarely alive one year after presentation. Due to the lack of any effective treatment for metastatic melanoma novel treatment strategies are urgently needed.

## **Part Two: Purinergic signalling**

Extracellular adenosine 5'-triphosphate (ATP) and other nucleotides are now firmly established as signalling molecules with important physiological and pathophysiological roles in many body systems. Their effects are mediated through a large group of cell surface receptors with varied roles. ATP acting on these receptors is involved with both rapid signalling in neurotransmission and also long term signalling in cell proliferation, differentiation and apoptosis.

### **1.2.1. Historical overview**

The physiological effects of extracellular nucleotides was first recognised in the cardiovascular system 75 years ago (Drury and Szent-Györgyi 1929). Further early studies confirmed the role of nucleotides in controlling heart rate, blood pressure

and vasodilation in the cardiovascular system; along with hints for the existence of multiple subtypes of purinergic receptors (Gillespie 1934).

The concept that ATP was an extracellular signalling molecule, as well as having an intracellular role in cell metabolism and as an energy source, took a long time to be accepted. In 1972, Burnstock proposed that ATP was a transmitter involved in non-adrenergic, non-cholinergic nerve mediated responses of the smooth muscle in the gastrointestinal tract and the bladder (Burnstock 1972). With the development of new research tools, such as molecular cloning, purinergic signalling has become an established principle not only in rapid signalling in neurotransmission (Dunn et al. 2001) but also in a wide range of other tissues and biological processes (Abbracchio and Burnstock 1998b;Ralevic and Burnstock 1998;Burnstock and Williams 2000). These include cell proliferation, differentiation and apoptosis in tissues as diverse as the skin, skeletal muscle, vessels and the immune system (Burnstock 2002b;Coutinho-Silva et al. 1999;Greig et al. 2003d;Ryten et al. 2002).

In the 30 years since purinergic signalling was first proposed, the concept of the purinergic receptor has been transformed from an idea into a therapeutic target in clinical practice (Burnstock 2002a;Burnstock 2003).

### **1.2.2. Classification of extracellular receptors for ATP**

Many receptors for extracellular nucleotides have been cloned in mammalian species. Purinoreceptors were originally classified on the basis of pharmacology and function. In 1978 Burnstock proposed the division of purinergic receptors into P1 receptors with adenosine as the main ligand and P2 receptors with ATP and adenosine 5'-diphosphate (ADP) as the main ligands (Burnstock 1978). P2 receptors were later subdivided into P2X and P2Y subtypes (Burnstock and Kennedy 1985). Despite the



re-classification of receptors on the basis of molecular structure this classification has, in principle, remained true (Abbracchio and Burnstock 1994). Currently purinoreceptors are classified as either P1, P2X or P2Y receptors (Burnstock 2003;Ralevic and Burnstock 1998).

The P1 receptor family consists of four receptors, A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub>, cloned and characterised in a variety of species. All of these receptors are coupled to G-proteins to inhibit (A<sub>1</sub> and A<sub>3</sub>) or stimulate (A<sub>2A</sub> and A<sub>2B</sub>) adenylate cyclase, thus reducing or increasing cAMP formation (Fredholm et al. 2001). P2X receptors constitute the most recently cloned family of ligand-gated ion channels. These receptors, which are gated by ATP, are non-selective cation channels permeable to Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup>. Seven receptor subtypes have been cloned: P2X<sub>1-7</sub>. These receptors are widespread and play several different physiological roles (Khakh et al. 2000). P2Y receptors are members of the superfamily of G-protein coupled receptors. To date eight receptors have been cloned in human cells. These receptors are highly diverse in their amino acid sequences and their profiles for endogenous ligands (Abbracchio et al. 2003;King et al. 2000).

### **1.2.3. P2X receptors**

It is becoming increasingly evident that P2X receptors are widely expressed throughout the body. *In situ* hybridisation, immunohistochemistry and functional studies have demonstrated that P2X receptors are expressed not only in excitable cells such as neurons and smooth muscle (Dunn et al. 2001), but also non-excitabile cells such as epithelia cells of the skin (Greig et al. 2003d), lungs (Wegner 2001) and kidneys (Unwin et al. 2003), as well as in bone (Hoebertz et al. 2003) and immune



cells (Burnstock 2001;Di Virgilio et al. 2001). P2X receptors are formed by P2X receptor subunits (Fig. 1.8A).

Figure 1.8. P2 receptor structure

Figure 1.8A Structure of P2X receptor subunit

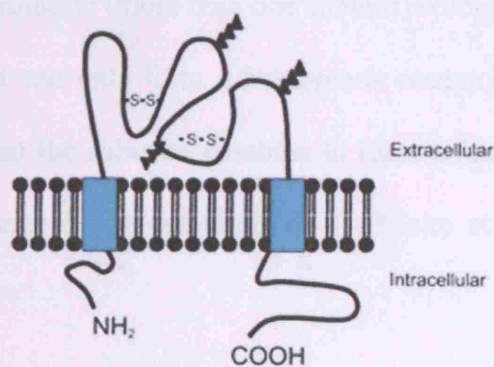


Figure 1.8B Multimeric P2X ion channel

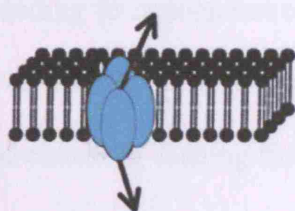
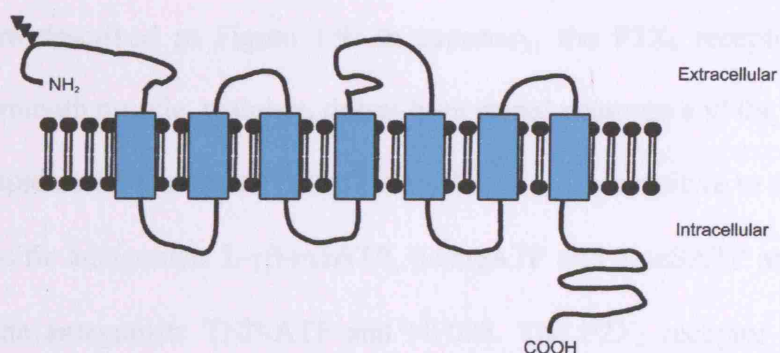


Figure 1.8C Structure of P2Y receptor



Figures modified from Burnstock 2006

Each subunit consists of two transmembrane domains, separated by an extensive N-glycosylated extracellular loop, always containing ten cysteine residues, and intracellular amino (N) and carboxy (C) termini. To date seven distinct P2X receptor subunits have been described: P2X<sub>1-7</sub>. These can form either homomeric (only one receptor subtype) or heteromeric (more than one subunit) receptors with the exception of the P2X<sub>7</sub> receptor that can only form a homomeric receptor (Torres et al. 1999). Recent studies suggest that the subunits combine to form a functional channel which allows ligand-gated passage of cations (Fig. 1.8B) (Nicke et al. 1998; Stoop et al. 1999).

P2X receptors are non-selective cation channels. Under normal physiological conditions, P2X receptor activation will result in to Na<sup>+</sup> and Ca<sup>2+</sup> influx and K<sup>+</sup> efflux across the cell membrane leading to depolarisation of the plasma membrane and an increase in intracellular to Na<sup>+</sup> and Ca<sup>2+</sup> concentration. Membrane depolarisation can in turn activate voltage-gated channels, causing firing of action potentials.

The pharmacological properties and tissue distribution of the P2X receptors have been recently reviewed (Burnstock and Knight 2004; Ralevic and Burnstock 1998) and are described in Figure 1.9. In summary, the P2X<sub>1</sub> receptor has been described in smooth muscle, platelets, dorsal horn spinal neurones and the cerebellum. It mediates rapid desensitization of the cell membrane and is sensitive to the ATP and the more specific antagonists L-γβ-meATP, βαmeATP and 2meSATP and it can be blocked by the antagonists TNP-ATP and NF023. The P2X<sub>2</sub> receptor is found in smooth muscle, the central nervous system, the retina, chromaffin cells and autonomic and sensory ganglia. Agonists are ATP, ATPγS, 2mSATP and βαmeATP; described antagonists acting at this receptor are Suramin and PPADS. The P2X<sub>3</sub> receptor has mainly been described in sensory and sympathetic neurones as well as more recently

Figure 1.9 P2X receptor subtypes

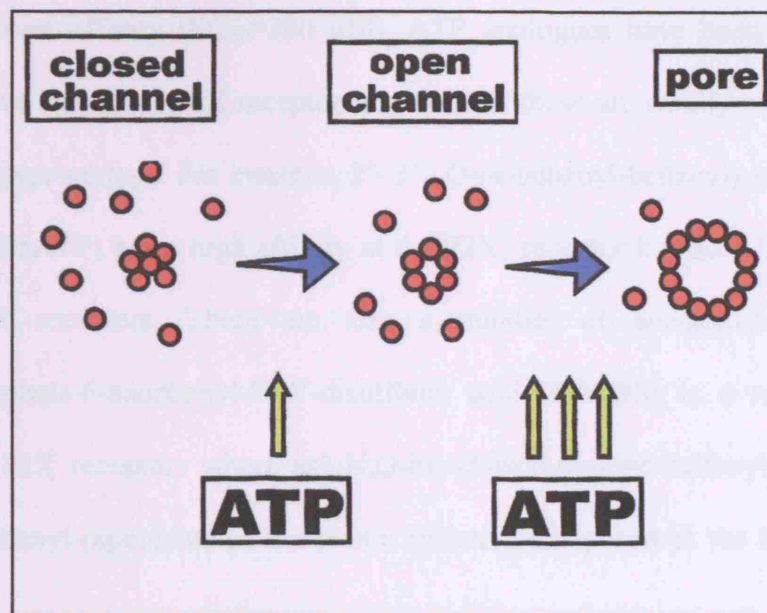
Receptor	Main Distribution	Agonists	Antagonists	Transduction Mechanisms
<b>P2X<sub>1</sub></b>	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurones	L- $\beta$ -meATP $\geq \alpha\beta$ meATP = ATP = 2meSATP rapid desensitization	TNP-ATP, NF023	intrinsic cation channel (Ca <sup>2+</sup> and Na <sup>+</sup> )
<b>P2X<sub>2</sub></b>	smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia	ATP $\geq$ ATP $\gamma$ S $\geq$ 2mSATP $\gg \alpha\beta$ meATP	Suramin, PPADS	intrinsic ion channel particularly Ca <sup>2+</sup>
<b>P2X<sub>3</sub></b>	sensory neurones, some sympathetic neurones	2meSATP $\geq$ ATP $\geq \alpha\beta$ meATP rapid desensitization	TNP-ATP, A317491, suramin, PPADS	intrinsic cation channel
<b>P2X<sub>4</sub></b>	CNS, Testis, colon	ATP $> \alpha\beta$ meATP		intrinsic ion channel (especially Ca <sup>2+</sup> )
<b>P2X<sub>5</sub></b>	Differentiating cells in skin, gut, bladder, thymus, spinal cord and skeletal muscle	ATP $\gg \alpha\beta$ meATP	Suramin, PPADS	intrinsic ion channel
<b>P2X<sub>6</sub></b>	CNS, motor neurones in spinal cord	does not function as homomultimer		intrinsic ion channel
<b>P2X<sub>7</sub></b>	Apoptotic cells in immune cells, pancreas, skin etc.	BzATP $>$ ATP $\geq$ 2meSATP $> \alpha\beta$ meATP	KN62, KN04 Coomassie Brilliant Blue G	intrinsic cation channel and a large pore with prolonged activation

in epithelia cells of the thyroid gland and keratinocytes in the epidermis of the skin (Denda et al. 2002; Glass and Burnstock 2001). Known agonists at this receptor are 2meSATP, ATP and  $\beta$ omeATP and antagonists are TNP-ATP, A317491, suramin and PPADS. The P2X<sub>4</sub> receptor has been demonstrated in the central nervous system, the testis and the colon. Agonists are ATP and  $\beta$ omeATP; no useful antagonists have been described. The P2X<sub>5</sub> receptor has a role in cell differentiation as well as short term signalling (Ryten et al. 2002), it has been found in cells in the skin, gut, bladder, thymus, skeletal muscle and the spinal cord. Described agonists are ATP, ATP $\gamma$ S and BzATP and antagonists are suramin and PPADS. The P2X<sub>6</sub> receptor does not function as a homomultimer but only as a heteromultimer with the P2X<sub>1-5</sub> receptor subunits. It is found in the central nervous system and motor neurones in the spinal cord.

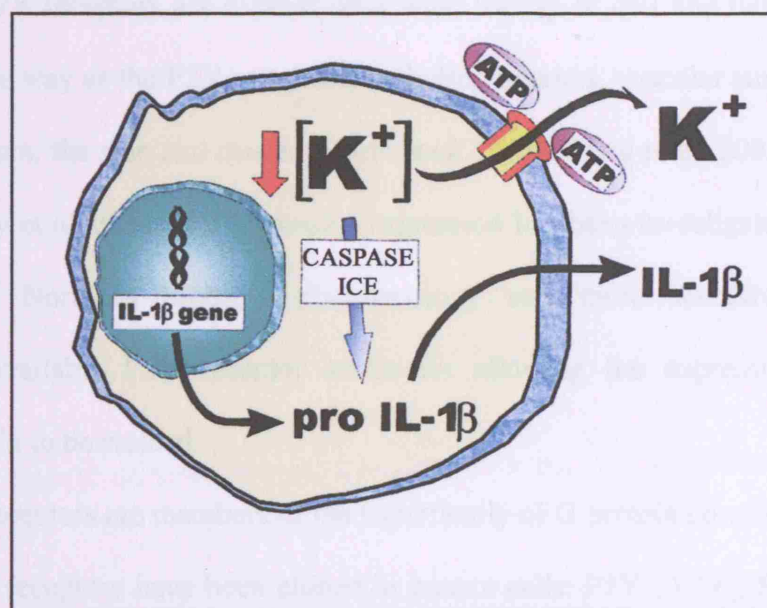
The P2X<sub>7</sub> receptor, which first cloned from rat brain and from macrophages in 1996 (Surprenant et al. 1996), differs in that, as well as functioning like other P2X receptors, when exposed at high concentration or for a long period to ATP, the cation channel can be converted to a large non-selective transmembrane pore which allows the passage of not only cations but small molecules up to the size of 900 daltons (Ralevic and Burnstock 1998) (figure 1.10a). This effect is associated with apoptosis mediated through the caspase enzyme system (Humphreys et al. 2000) and interleukin-1 $\beta$  release (Solle et al. 2001) (figure 1.10b). P2X<sub>7</sub> receptors have been described in a wide range of human tissues and cells including leukocytes (Dubyak 2000), hepatocytes (Keppens 1993) and keratinocytes (Greig et al. 2003d).

Currently no selective agonists exist for all the purinoreceptors (Burnstock 2003; Jacobson et al. 2000) and therefore pharmacological identification relies on the effects of a combination of compounds. ATP itself is active over the entire range of P2X receptors although it has different efficacies at each receptor (Khakh et al. 2000),

Figure 1.10 The P2X<sub>7</sub> receptor



(A) The P2X<sub>7</sub> receptor is generated by the aggregation of a number of subunits to form an ATP-activated channel. Recruitment of additional subunits by a high concentration of ATP causes formation of a non-selective pore.



(B) Opening of the P2X<sub>7</sub> pore by extracellular ATP causes a large K<sup>+</sup> efflux that triggers proteolytic activation of caspase and interleukin converting enzyme (ICE) which causes cleavage of interleukin precursor molecules and leads to cytokine release and apoptosis.

Figure 1.10B modified from Di Virgilio et al 2001

with P2X<sub>3</sub> receptors having the highest affinity ( $EC_{50}=1\text{ }\mu\text{M}$ ) and P2X<sub>7</sub> receptors having the lowest affinity ( $EC_{50}=300\text{ }\mu\text{M}$ ). ATP analogues have been synthesised which are active ligands at P2X receptors but none of these are wholly selective for a particular receptor subtype. For instance, 2'-3'-O-(4-benzoyl-benzoyl) adenosine 5'-triphosphate (BzATP) has a high affinity at the P2X<sub>7</sub> receptor but does have activity at other P2X receptors. There are also a number of antagonists available. Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) is a non-selective antagonist at P2X receptors where as 1-N,O-bis-[5-isoquinoline-sulfonyl]-N-methyl-L-tyrosyl)-4-phenyl-piperazine (KN-62) is a selective antagonist at the human P2X<sub>7</sub> receptor.

#### 1.2.4. P2Y receptors

The P2Y receptors are expressed on a wide variety of cell and tissue types, in much the same way as the P2X receptors, including neurons, vascular smooth muscle and endothelium, the skin and cancers (Burnstock 2002b; Greig et al. 2003d; Katzur et al. 1999; Neary et al. 1996). P2Y receptor expression has been investigated by *in situ* hybridisation, Northern blotting, pharmacology and more recently with the commercially available P2Y receptor antibodies allowing the expression of P2Y receptor protein to be studied.

P2Y receptors are members of the superfamily of G-protein coupled receptors. To date eight receptors have been cloned in human cells: P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub>. These receptors are highly diverse in their amino acid sequences and their profiles for endogenous ligands (King et al. 2000). P2Y receptors contain seven transmembrane domains, with an extracellular N-terminus and intracellular C-terminus (Fig 1.8C). Several secondary messenger transduction

pathways have been implicated in P2Y receptor signalling. These include phospholipase C (PLC) activation, adenylate cyclase inhibition and stimulation and direct modulation of ion channel function. Activation of PLC leads to the formation of inositol 1,4,5-triphosphate (IP<sub>3</sub>) and mobilisation of Ca<sup>2+</sup> from intracellular stores such as the endoplasmic reticulum.

P2Y receptor distribution and pharmacology is summarised in figure 1.11. P2Y receptors have been shown to have many diverse roles. Within the nervous system they have been shown to modulate transmitter release from neurons (Bennett et al. 2003). P2Y receptors also play a significant role in the cardiovascular system causing vessels to dilate via endothelial-dependent and independent pathways and to mediate direct muscle constriction of some vessels (Ralevic and Burnstock 1998). The most established role of P2Y receptors in clinical practice is the control of platelet aggregation, and clinical trials have shown that drugs such as clopidogrel (a P2Y<sub>12</sub> antagonist) reduces the risks of strokes and heart attacks (Yusuf et al. 2001). P2Y<sub>2</sub> receptors have also been implicated in the regulation of Cl<sup>-</sup> conductance and mucus secretion by airway epithelial cells (Cressman et al. 1999). As a result the use of aerosolised nucleotides for the treatment of cystic fibrosis and chronic bronchitis is being investigated (Burnstock 2002a). Perhaps the most significant recent role of P2Y receptors to be demonstrated is their long term trophic effects on cell proliferation. Activation of the P2Y<sub>2</sub> receptor has been shown to increase the proliferation of astrocytes and keratinocytes (Greig et al. 2003d; Neary 2000) while the P2Y<sub>1</sub> receptor mediates a decrease in astrocytoma cells (Sellers et al. 2001).

Much progress has been made in characterising the pharmacological profile of the P2Y receptors but, as in the case with the P2X receptors, there are only a limited number of selective and specific agonists and antagonists. P2Y<sub>1</sub>, P2Y<sub>12</sub> and P2Y<sub>13</sub>

Figure 1.10 P2Y receptor subtypes

Receptor	Main Distribution	Agonists	Antagonists	Transduction Mechanisms
<b>P2Y<sub>1</sub></b>	Epithelial and endothelial cells, platelets, immune cells, osteoclasts	2meSADP >2meSATP = ADP >ATP	MRS2279 MRS2179	Gq/G11 PLC $\beta$ activation
<b>P2Y<sub>2</sub></b>	Immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts	UTP =ATP	Suramin	Gq/G11 and Gi PLC $\beta$ activation
<b>P2Y<sub>4</sub></b>	Endothelial cells	UTP $\geq$ ATP	Reactive Blue 2 PPADS	Gq/G11 and Gi PLC $\beta$ activation
<b>P2Y<sub>6</sub></b>	Some epithelial cells, placenta, T-cells, thymus	UDP >>UTP >>ATP	Reactive Blue 2 PPADS suramin	Gq/G11 PLC $\beta$ activation
<b>P2Y<sub>11</sub></b>	Spleen, intestine, granulocytes	ARC67085MX >BzATP $\geq$ ATP $\gamma$ S >ATP	Suramin Reactive Blue 2	Gq/G11 and GS PLC $\beta$ activation
<b>P2Y<sub>12</sub></b>	Platelets, glial cells	ADP >>ATP	ARC67085MX ARC69931MX	Gi/Go inhibition of adenylate cyclase
<b>P2Y<sub>13</sub></b>	Spleen, brain, lymph nodes, bone marrow	ADP =2meSADP >>ATP +2meSATP		Gi/Go
<b>P2Y<sub>14</sub></b>	Placenta, adipose tissue, stomach, intestine	UDP glucose = UDP-galactose		Gq/G11



receptors are selectively activated by ADP, while P2Y<sub>2</sub> and P2Y<sub>4</sub> receptors respond to UTP with an equal potency to ATP. P2Y<sub>6</sub> receptors are selective for UDP. The P2Y<sub>1</sub> receptor can be selectively activated using the synthetic agonist 2-methylthioadenosine-5-diphosphate (2MeSADP). Activation of all the P2Y receptors, with the exception of P2Y<sub>2</sub>, can be inhibited by the non-selective antagonists pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) and suramin. N<sup>6</sup>-methyl-2'-deoxyadenosine 3',5'-bisphosphate (MRS 2179) is a selective antagonist at the P2Y<sub>1</sub> receptors while clopidogrel is a selective antagonist at the P2Y<sub>12</sub> receptor (King et al. 2000;Ralevic and Burnstock 1998).

#### **1.2.5. Sources and fate of extracellular nucleotides**

The level of extracellular ATP reflects a balance between ATP release from cells and its extracellular degradation. Nucleotides are passively released from damaged and dying cells, the intracellular concentration of ATP is 2-5 mM and damage to the cell membrane can cause the local release of ATP (Chow et al. 1997). During trauma nucleotides have been shown to obtain local extracellular levels as high as 20 µM (Born and Kratzer 1984), a concentration sufficient to activate all P2 receptor subtypes.

ATP is also actively secreted from cells in a number of ways. Exocytosis of vesicles containing ATP is a mechanism that has been described in both nerves and vascular endothelial cells (Bodin and Burnstock 2001b;Bodin and Burnstock 2001a). In nerve terminals ATP is often stored with other neurotransmitters in synaptic vesicles, which upon depolarisation are released in a Ca<sup>2+</sup>-dependent manner by membrane/vesicle fusion.

There are also local (paracrine and autocrine) mechanisms of release. The release of ATP from cells occurs through cell membrane complexes such as the ATP-binding cassette (ABC) proteins. These ABC proteins include the cystic fibrosis conductance regulator (CFTR), P-glycoprotein and the sulfonylurea receptor (Abraham et al. 1997). The existence of mechanosensitive ATP channels that can mediate ATP release induced by mechanical stimuli such as mechanical strain and hypotonic swelling has been described (Grygorczyk and Hanrahan 1997; Sauer et al. 2000; Wang et al. 1996). There is also the possibility that ectonucleotidases, which normally degrade ATP, might synthesise ATP by a backward phosphorylation reaction ( $\text{AMP} \rightarrow \text{ADP} \rightarrow \text{ATP}$ ) (Yegutkin et al. 2001). This report suggests that, depending on the local nucleotide concentration, adenine nucleotides on the cell surface can undergo both hydrolysis and resynthesis.

The action of extracellular nucleotides at P2 receptors is terminated by cell surface enzymes that sequentially degrade nucleoside 5'-triphosphates to their respective nucleotides and phosphate. These enzymes are the ectonucleotidases, they form a large family of membrane-bound enzymes and included ectonucleoside triphosphate diphosphohydrolase, alkaline phosphatase and ecto-5'-nucleotidase (Kaczmarek et al. 1996; Ujhazy et al. 1996). A feature of this hydrolysis cascade is that the breakdown products (nucleoside diphosphates, monophosphates and nucleotides) all appear in the extracellular fluid and may also be active at purinergic receptors (Zimmermann 2001).

## **Part Three: Purinergic signalling in melanoma**

Recently the possibility of purinergic signalling in the skin cancer melanoma has been suggested. Purinergic signalling in both the skin and cancer are rapidly expanding fields which are discussed in this section followed by a review of existing reports on purinergic signalling in malignant melanoma.

### **1.3.1. Purinergic signalling in the skin**

Purinergic signalling plays an important role in the skin. The skin is a complex organ containing many cell types and a role for both short term, fast acting and long term, slower acting purinergic signalling has been described in a variety of these cell types. These include keratinocytes and melanocytes in the epidermis, fibroblasts in the dermis and cutaneous vessels.

The majority of research into purinergic signalling in the skin has been performed in keratinocytes. Previous work has shown adenosine and ATP have an anti-proliferative effect on cultured human keratinocytes and that the adenosine A<sub>2B</sub> receptor mRNA is present in human keratinocytes (Brown et al. 2000;Cook et al. 1995). P2 receptors were first described in rat epidermis (Groschel-Stewart et al. 1999). In human tissues and cells in culture P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors have been demonstrated to be involved in cell proliferation, P2X<sub>3</sub> receptors have been described with a role in barrier repair, P2X<sub>5</sub> receptors are in differentiating keratinocytes and the P2X<sub>7</sub> receptor regulates apoptosis in these cells (Burrell et al. 2003;Denda et al. 2002;Dixon et al. 1999;Greig et al. 2003d). P2 receptors also have been shown to be involved in the development of the fetal epidermis (Greig et al. 2003b), healing in a

model of an epidermal wound (Greig et al. 2003a) and to be expressed in cancers of keratinocyte origin (Greig et al. 2003c).

The only description of the possible role of purinergic signalling in pigment producing cells, such as melanocytes, is research in lower vertebrates. Many fish are capable of spectacular colour changes due to the motile activities of pigmented cells called melanophores or melanosomes rather than melanocytes. These are controlled by both nerves and hormones, including melanophore-stimulating hormone secreted from the intermediate lobe of the pituitary gland which gives rise to a darkening in skin colour, which is antagonised by melanin concentrating hormone which causes blanching. In both catfish and damselfish the darkening reaction is antagonised by methylxanthines (P1 antagonists) suggesting an adenosine receptor is involved in the regulation of melanophores (Kasukawa et al. 1985; Miyashita et al. 1984). In more recent studies of denervated melanophores in the medaka fish it was concluded that an A<sub>2</sub> receptor subtype was mediating the response (Namoto 1987). Evidence that ATP is released as a cotransmitter together with noradrenaline from melanosome aggregating sympathetic nerves in the tilapia fish has been presented (Kumazawa and Fujii 1984). It seems likely that ATP released from sympathetic nerves is broken by ectoenzymes to adenosine which then acts on P1 receptors both on melanophores and on prejunctional sites to modulate transmitter release. No descriptions of P2 receptors in pigmented cells in other species or any purinergic signalling mechanism in human melanocytes exist.

Fibroblasts are dermal cells which produce collagen and are key to healing wounds in the skin. The presence of P1 receptors (Brackett and Daly 1994; Murakami et al. 2001) and P2 receptors (Solini et al. 1999; Solini et al. 2000) have been described in fibroblasts using RT-PCR, western blots and pharmacological

characterisation. Adenosine has been shown to increase the speed of wound healing (Montesinos et al. 1997) by acting at P1 receptors using a variety of experimental techniques including a A<sub>2A</sub> receptor knockout mouse (Montesinos et al. 2002).

Purines have a role to play in the control of cutaneous vascular tone. ATP, ADP and AMP have vasodilatory effects on the artery supplying the facial skin when infused intra-arterially (Bari et al. 1993). Vasoconstricting P2X receptors and vasodilating P2Y and P1 receptors have been identified in the subcutaneous arterial bed (Martin et al. 1991). ATP is released from both perivascular sympathetic nerves with noradrenaline (Kennedy et al. 1986) and vascular endothelial cells during changes in flow and hypoxia to act on P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors, leading to the production of nitric oxide and subsequent vasodilation (Burnstock 2002b).

### **1.3.2. Purinergic signalling in cancer**

The anticancer activity of adenine nucleotides was first described in 1983 (Rapaport et al. 1983). Since then ATP has been shown to have a variety of effects on cancer, including improvement of cancer cachexia, inhibition of tumour growth and a synergistic action when administered with established chemotherapeutic agents. The study of purinergic signalling in cancer has focused in four areas; these are the *in vitro* study of cancer cell lines, immunohistological examination of excised specimens of cancers, *in vivo* animal models and clinical trials.

ATP has been shown to inhibit the growth of human cell lines of numerous cancer types which include: oesophageal cancer (Maaser et al. 2002), endometrial cancer (Katzur et al. 1999), lung cancer (Schafer et al. 2003), colorectal cancer (Hopfner et al. 1998), gliomas (Czajkowski et al. 2002), prostate cancer (Janssens and

Boeynaems 2001) and basal cell and squamous cell carcinoma of the skin (Greig et al. 2003c). Attempts have been made to identify the mechanism of action of ATP on cancer cells and the purinergic receptor subtypes involved and these cell lines have been studied using a variety of techniques to show the expression of P2 receptor protein (immunocytochemistry and western blots), mRNA (RT-PCR) and functional responses to ATP (intracellular calcium levels). Several P2 receptors have been implicated in these studies, they are the metabotropic P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors and the ionotropic P2X<sub>5</sub> and P2X<sub>7</sub> receptors. Alteration of cancer cell number may be due to mediation of cell proliferation via P2Y receptors, stimulation of differentiation with subsequent inhibition of proliferation via P2X<sub>5</sub> receptors or induction of cell death (apoptosis) via P2X<sub>7</sub> receptors. However P2Y receptors may mediate opposite actions in different cancer types. For instance, P2Y<sub>2</sub> receptors have been shown to mediate an increase in cell number in lung cancer (Schafer et al. 2003) and squamous cell carcinoma of the skin (Greig et al. 2003c), but cause a decrease in proliferation in endometrial (Katzur et al. 1999) and colorectal (Hopfner et al. 1998) cancer cell lines. The concentrations of ATP and other purinergic receptor agonists needed to effect a change in cell number varies between cell type and experimental design but is within the range 30  $\mu$ M to 3mM. In addition to its isolated action on cell number the synergistic action of ATP when combined with conventional cancer chemotherapy such as doxorubicin caused a doubling of cell mortality in human ovarian carcinoma cells (Maymon et al. 1994).

P2 receptors have been demonstrated using immunohistochemistry in formalin-fixed, paraffin-embedded, cancer specimens that have been excised from patients. The P2X<sub>7</sub> receptor has been demonstrated in basal cell and squamous cell carcinoma of the skin (Greig et al. 2003c), prostate cancer (Slater et al. 2003b) and

breast cancer (Slater et al. 2004). P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2X<sub>5</sub> receptors have also been described in basal cell and squamous cell carcinoma of the skin (Greig et al. 2003c). It has been postulated that purinergic receptor expression can be used as a marker of cancer prognosis, with for example the up-regulated expression of the apoptotic P2X<sub>7</sub> receptor as a sign of premalignant change.

In the original *in vivo* studies of treating cancer with ATP it was shown that daily intraperitoneal injection of 1-2 mg ATP per g body weight, resulted in a circulating plasma level of 5-10  $\mu$ M ATP 4-8 hours post injection, which significantly inhibited tumour growth in mice with CT26 colon tumours (Rapaport 1988; Rapaport and Fontaine 1989; Rapaport 1990). ATP has also been demonstrated to inhibit tumour growth in mice with lymphomas (Nayak et al. 1990), Ehrlich ascites tumours (Lasso de la Vega MC et al. 1994), fibrosarcomas (Froio et al. 1995) and breast tumours (Abraham et al. 1996). The intraperitoneal inject of ATP also significantly prolonged the survival time of the *in vivo* models (Estrela et al. 1995; Obrador et al. 1997).

The study of purinergic signalling in cancer and whether ATP can be used as a therapeutic agent has led to preliminary clinical trials. Three such trials have been reported to date, which treated small groups of patients with advanced cancer; mainly inoperable non-small cell lung cancer (Agteresch et al. 2003; Haskell et al. 1996; Haskell et al. 1998). Patients received between 1 to 10 infusions of intravenous ATP at a dose of 20-100  $\mu$ M/Kg. Although no significant tumour shrinking was observed, the majority of patients exhibited stable disease after treatment with ATP. In addition, beneficial effects were seen in terms of overall survival and weight gain (decreased cachexia). Side effects were however seen at high doses relating to cardiovascular disturbances such as headaches, flushing, chest tightness and dyspnea.

### 1.3.3. Purinergic signalling in melanoma

The study of purinergic signalling in melanoma has been largely confined to P1 (adenosine) rather than P2 (ATP) receptors. In 1998 a role for an adenosine receptor mediating melanoma cell motility in the human melanoma cell line A2058 was described and it was proposed that this was mediated by the A<sub>1</sub> receptor subtype (Woodhouse et al. 1998). In 2001 selective activation of the A<sub>3</sub> receptor was described as having an anti-proliferative effect on the murine B-16 melanoma cell line *in vitro* and *in vivo* (Fishman et al. 2001). Two further reports suggested the presence of all 4 P1 receptor subtypes in the human melanoma A375 cell line and described a role for the A<sub>2A</sub> receptor in inducing cell death and attributing the A<sub>3</sub> receptor with stimulating cell growth (Merighi et al. 2001; Merighi et al. 2002).

In contrast to the conflicting reports concerning P1 receptor function in melanoma, P2 receptor function has been poorly studied. The P2X<sub>7</sub> receptor has been described in a single report investigating changes in apoptotic markers in keratinocytes at the edges of formalin-fixed, paraffin-embedded melanoma specimens, using only the single technique of immunohistochemistry (Slater et al. 2003a). In the mouse melanoma cell line M3 an *in vitro* application of ATP additively potentiated the cytotoxic effects of various chemotherapeutic agents such as flurouracil, doxorubicin, mitomycin and vincristine (Mure et al. 1992). The mechanism of action of ATP was not however investigated. The field of P2 receptor signalling remains to be investigated to see whether these purinergic receptors are a target for the treatment of malignant melanoma.



## **Chapter Two**

# **P2Y purinergic receptors regulate the growth of human melanomas**

## **2.1. Abstract**

Adenosine 5'-triphosphate is known to function as a potent extracellular messenger producing its effects via a distinct family of cell surface receptors. Different receptor subtypes have been shown to modulate different cellular functions such as proliferation, differentiation and apoptosis. We investigated the functional expression and proliferative action of metabotropic P2Y receptors in human melanoma tissue and cells. Expression of functional P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>6</sub> receptor subtypes was established by reverse transcriptase polymerase chain reaction, immunohistochemistry and intracellular calcium measurements using a Fluorometric Imaging Plate Reader. Incubation of A375 melanoma cells with the P2Y<sub>1</sub> receptor-selective agonist 2-methylthioadenosine-5-diphosphate caused a decrease in cell number which was dose dependent, whereas incubation with the P2Y<sub>2</sub> receptor agonist uridine triphosphate caused a dose dependent increase in cell number. The action of extracellular nucleotides on P2Y receptors was shown to mediate the growth of melanomas and the P2Y<sub>1</sub> receptor is a putative target for melanoma therapy.

## **2.2. Introduction**

Melanoma is an important cutaneous malignancy as it accounts for 95% of all deaths from skin disease (Dreiling et al. 1996). The incidence of melanoma is increasing (Osborne 2002) and the outcome for patients with advanced disease remains poor as there is resistance to current methods of treatment (Serrone and Hersey 1999), therefore new treatment strategies are needed.

There is increasing evidence that purinergic signalling can have prolonged effects on cell growth and proliferation (Burnstock 2002b; Greig et al. 2003d). The current classification of purinergic receptors, suggested by Burnstock in 1978 (Burnstock 1978), divides purinergic receptors into two groups. P1 receptors are selective for adenosine (Fredholm et al. 2001) while P2 receptors are selective for adenosine 5'-triphosphate (ATP) (Ralevic and Burnstock 1998), P2 receptors are further subclassified by their pharmacological properties and transduction mechanisms into P2X and P2Y receptors (Abbracchio and Burnstock 1994; Burnstock and Kennedy 1985).

P2X receptors are ligand gated ion channels which are activated by extracellular ATP to elicit a flow of cations (Khakh et al. 2000). Seven subtypes of these ionotropic receptors have been identified (P2X<sub>1</sub>-P2X<sub>7</sub>). P2X receptors are mainly expressed in the nervous system, platelets and in smooth muscle cells. P2Y receptors, however, belong to the family of G-protein coupled receptors (King et al. 2000) and the principal signal transduction pathway involves phospholipase C, which leads to the formation of inositol 1,4,5-triphosphate (IP<sub>3</sub>) and mobilization of intracellular calcium (Berridge 1987). Depletion of intracellular calcium stores leads to calcium mediated calcium influx across the cell membrane (Berridge 1995). Metabotropic P2Y receptors have been described in a wide range of normal tissues

including blood vessels (Burnstock 2002b), bone (Hoebertz et al. 2003), the gastrointestinal tract (Roman and Fitz 1999) and the epidermis (Greig et al. 2003d).

The anti-cancer activity of adenine nucleotides was first described by Rapaport in 1983 (Rapaport et al. 1983). Intraperitoneal injection of ATP into tumour bearing mice resulted in significant anti-cancer activity against several fast growing, aggressive carcinomas (Rapaport and Fontaine 1989). The presence of P2Y receptors has been described in several cancer types including oesophageal (Maaser et al. 2002), prostate (Janssens and Boeynaems 2001), lung (Schafer et al. 2003), sarcoma and colorectal cancer cell lines (Nylund et al. 2004). Both P1 (Merighi et al. 2001; Merighi et al. 2002) and P2X<sub>7</sub> receptors (Slater et al. 2003) have previously been described in melanoma. P2Y receptors have not been examined in melanomas to date. Therefore we investigated the role of P2Y receptor mediated effects of extracellular nucleotides in human melanoma tissue and cells.

## **2.3. Materials and Methods**

### **2.3.1. Materials**

The melanoma cell line A375 (Giard et al. 1973) was obtained from the Wellcome Trust Functional Genomics Cell Bank (St Georges Hospital Medical School, London, UK). Cell culture medium and reagents were purchased from Sigma (Poole, UK). SV Total RNA Isolation system was purchased from Promega (WI, USA) and Ready-To-Go RT-PCR beads were purchased from Amersham Biosciences (New Jersey, USA). RT-PCR primers were obtained from Invitrogen (Paisley, UK).

### **2.3.2. Cell culture**

Melanoma cells were grown in 90% Dulbecco's Modified Eagle's Medium (DMEM) and 10% heat inactivated fetal calf serum supplemented with penicillin (100 U per ml), streptomycin (100 µg per ml) and L-glutamine (2 mM) in 75 cm<sup>2</sup> tissue culture flasks (Corning, New York, USA). Cells were incubated at 37°C in 5% CO<sub>2</sub> /95% air and were subcultured at 70% confluence. Cell viability was determined using the trypan blue exclusion method.

### **2.3.3. Immunohistochemistry of paraffin embedded specimens of melanoma**

A total of 14 specimens of melanoma were examined for P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub> receptors. Paraffin blocks were sectioned at 4 µm on a Reichert-Jung Microtome, and sections were taken on Snowcoat Extra slides (Surgipath, Cambridgeshire, UK), then dried in an oven for 2 hours at 60°C. Sections were dewaxed and rehydrated using xylene and graded concentrations of ethanol. Antigen retrieval was performed by microwaving for 10 minutes in a solution of 1 mM ethylenediamine tetraacetic acid (Tris-EDTA) at pH 9.0. Endogenous alkaline

phosphatase was blocked by 20 minutes of incubation in 20% acetic acid. Sections were washed and then incubated with avidin D blocking solution, biotin blocking solution and 1:5 normal swine serum (Vector Laboratories).

Polyclonal anti-P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub> receptor antibodies, corresponding to a 14-16 peptide sequence of the intracellular portion of the transmembrane receptors were obtained from Alomone Laboratories (Jerusalem, Israel). They were kept frozen at a stock concentration of 0.6 mg/ml and used at a dilution of 1:100. 100 µl of anti-P2Y receptor antibody, diluted 1:100, was applied for 12 hours at 4°C. 100 µl of biotinylated anti-rabbit antibody (DAKO E0353), diluted 1:200 in DAKO ChemMate was applied for 30 minutes followed by 100 µl of streptavidin alkaline phosphatase (Vector SA5100) diluted 1:200 in DAKO ChemMate for 30 minutes. Vector Red substrate (Vector Alkaline phosphatase substrate, SK5100) made up in 200mM Tris-HCl (pH 8.2) was then applied for 10 minutes. Positive staining appeared bright pink, nuclei were counterstained with hematoxylin (purple). All sections were subsequently dehydrated, cleared and mounted. Negative controls were performed by either omission of the primary antibody or preabsorption of the primary antibody with the corresponding peptide sequence.

#### **2.3.4. Immunocytochemistry**

A375 cells were grown in culture on chamber slides (Nunc, Illinois, USA) until 50% confluent. They were fixed in 4 % formaldehyde in 0.1 M phosphate buffer for 2 minutes. Non-specific binding sites were blocked by a 20 minute preincubation with 10% normal horse serum (NHS) in 0.1 M phosphate buffer containing 0.05% merthiolate, followed by incubation with primary P2Y receptor antibody with 0.2%

Triton, for 12 hours at 4°C. Subsequently, the slides were incubated with donkey anti-rabbit Cy3 (Jackson ImmunoResearch, Pennsylvania, USA) diluted 1:300 with 1 % NHS in phosphate buffer. Slides were then mounted with Eukitt (BDH laboratories, Dorset, UK) and examined. Control experiments were carried out with the primary antibody being omitted from the staining procedure or the primary antibody preabsorbed with the corresponding peptide. All other reagents were obtained from Sigma (Poole, UK).

### **2.3.5. Reverse transcriptase polymerase chain reaction**

The expression of mRNA encoding the P2Y receptors in A375 melanoma cells was determined using reverse transcriptase (RT) and the polymerase chain reaction (PCR). Total RNA was isolated from A375 cells grown in culture using the SV Total RNA Isolation System. The RT-PCR reactions were performed using Amersham Biosciences Ready-To-Go RT-PCR beads. Reverse transcriptase was carried out on 1 µg of RNA for 30 minutes at 42°C. The polymerase chain reaction cDNA samples were initially denatured for 5 minutes at 95°C prior to initiating the PCR step. The P2Y primer sequences used were as follows: P2Y<sub>1</sub> 5'-cgg tcc ggg ttc gtc c-3' and 5'-cgg acc ccg gta cct-3' product size 527 base pairs; P2Y<sub>2</sub> 5'-ctc tac ttg gtc acc acc agc g-3' and 5'-ttc tgc tcc tac agc cga atg tcc-3' product size 637 base pairs; P2Y<sub>4</sub> 5'-cca cct ggc att gtc aga cac c-3' and 5' gag tga cca ggc agg gca cgc-3' product size 424 base pairs and P2Y<sub>6</sub> 5'-cgc ttc ctc ttc tat gcc aac c -3' and 5'-cca tcc tgg cgg cac agg cgg c -3' product size 364 base pairs as previously described (Adrian et al. 2000). The amplification reaction was conducted under the following conditions: 95°C for 30 seconds, the optimal annealing temperature for 30 seconds (54°C for P2Y<sub>1</sub> and P2Y<sub>4</sub>; 62°C for the P2Y<sub>2</sub> and P2Y<sub>6</sub>) and 72°C for 1 minute, run

for 35 cycles. Amplification products were separated by electrophoresis and visualised by ethidium bromide staining. The presence of possible contaminants was investigated in all using control RT-PCR reactions in which, either mRNA had been omitted, or heating to 95°C had inactivated the reverse transcriptase. Each reaction was repeated at least 3 times each from a separate mRNA preparation.

### **2.3.6. Measurement of intracellular calcium changes**

A375 human melanoma cells were grown in 96-well plates (BD Falcon, New Jersey, USA) to confluence. Cells were loaded with the fluorescent dye Fluo-4 AM (Molecular Probes, Leiden, The Netherlands) at a concentration of 2  $\mu$ M for 30 minutes at room temperature in a bath solution containing: 20 mM HEPES, 140 mM NaCl, 4 mM KCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{K}_2\text{HPO}_4$ , 1 mM  $\text{CaCl}_2$  and 10 mM D-Glucose with the pH adjusted to 7.4 with 1M NaOH. For the experiments with calcium free buffer, the  $\text{CaCl}_2$  was omitted and 0.1 mM EGTA was added. To remove excess dye that had not been taken up by the cells, plates were washed 3 times with bath solution. The plates were placed into a Fluorometric Imaging Plate Reader (FLIPR- Molecular Devices, Ca, USA) where they were incubated at 37°C, prior to pre-incubation with the purinergic antagonists pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) and N<sup>6</sup>-methyl-2'-deoxyadenosine 3',5'-bisphosphate (MRS 2179) and the addition of the P2Y agonists ATP, uridine triphosphate (UTP), uridine diphosphate (UDP) and 2-methylthioadenosine-5-diphosphate (2MeSADP). The cells and dye were excited by an argon-ion laser source at a wavelength of 488 nm and emission was measured at 520 nm at 1 second intervals. The change in fluorescence (F) measured was plotted as a change from average fluorescence ( $F_0$ ) prior to the addition of agonist.



### 2.3.7. Cell proliferation assay

Cells were seeded onto a 96 well plate at a density of 250 cells per well (2,500 cells per ml with 100  $\mu$ l of cell suspension per well). This seeding density gave the best growth curve over a 96 hour period. 24 hours after seeding the medium was aspirated and fresh medium containing either P2Y receptor agonist or just medium (control) was added to the well plates. ATP, 2-MeSADP, UTP, UDP were added in concentrations from 1  $\mu$ M to 100  $\mu$ M. The pH of the drug solutions prepared in the culture medium was adjusted to 7.36-7.44 prior to addition to the cells.

Changes in cell number were quantified by a colourimetric assay using crystal violet (Gillies, 1986) and read using a spectrophotometric plate reader (Labtech, East Sussex, UK) at 24, 48 and 72 hours after addition of purinergic agents.

For the colourimetric assay, a solution of 0.5 g crystal violet, 0.85 g NaCl, 5 ml 10% formal saline, 50 ml absolute ethanol, 45 ml distilled water was used. Medium was aspirated from the wells and 100  $\mu$ l of the colourimetric assay mixture was added to each well and incubated at room temperature for 10 minutes. This mixture allowed simultaneous fixation of cells and penetration of the crystal violet dye into the cells. After washing 3 times with phosphate buffered saline (PBS), 33% acetic acid was used to elute colour from the cells and optical density was read at 570 nm using the spectrophotometric plate reader. To confirm that the optical density of the wells correlated with cell number, a control assay was performed for each experiment where known numbers of cells were seeded in ascending seeding densities and the plate read as soon as the cells had attached. Cell number versus optical density was plotted. The  $R^2$  value of the trend line was always greater than 0.98.

### **2.3.8. Photography**

The results were analysed using a Zeiss Axioplan high definition light microscope (Oberkochen, Germany) mounted with a Leica DC 200 digital camera (Heerbrugg, Switzerland).

### **2.3.9. Statistical analysis**

Each experiment was repeated at least 3 times each with 3-8 samples. Data analysis was performed using Microsoft Excel XP Professional and GraphPad Prism 3.0 software. Comparisons of multiple means was performed by analysis of variance (ANOVA), the difference between samples was considered as significant when P-value was  $<0.05$ .

## **2.4. Results**

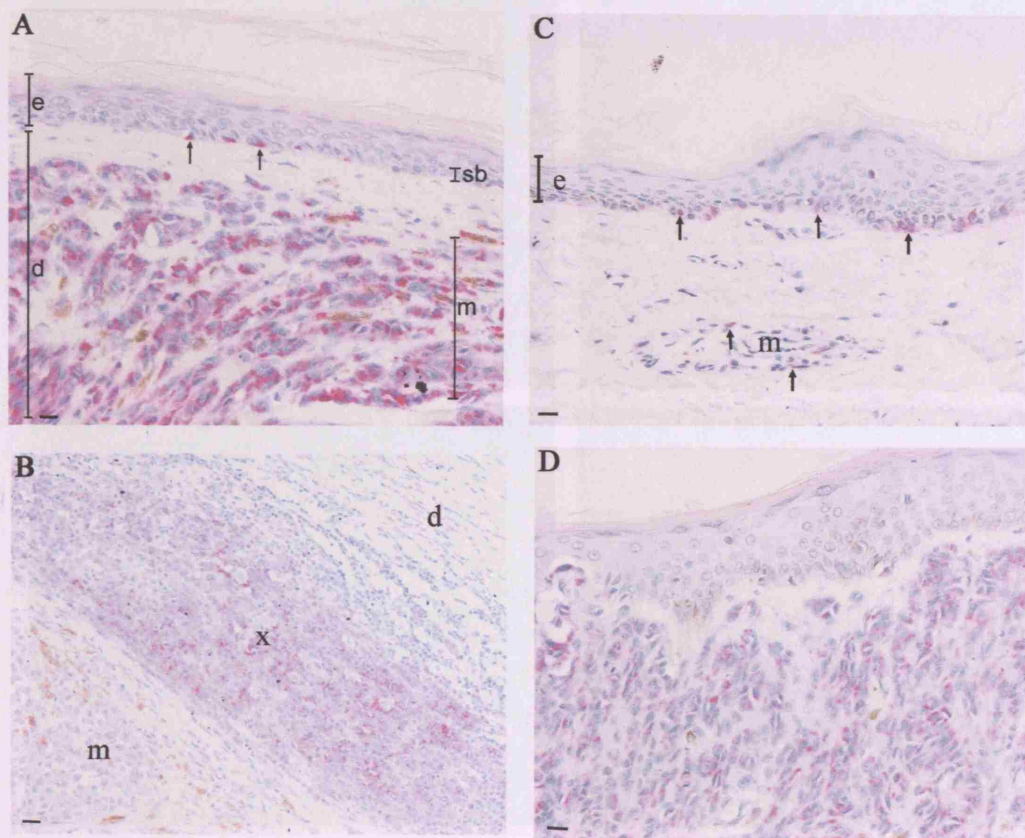
### **2.4.1. P2Y receptors are expressed in tissue sections of melanoma**

Positive labelling for P2Y receptor expression was present in all specimens of melanoma examined. The staining was uniform throughout with all melanoma cells appearing to express P2Y<sub>1</sub> and P2Y<sub>6</sub> receptors (Fig. 2.1A, Fig. 2.1D). P2Y<sub>2</sub> receptor staining was present, but was localised at the proliferating margins of the melanoma (Fig. 2.1B). No positive staining for P2Y<sub>4</sub> receptor protein was present in any of the specimens. As an internal positive control, P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors were also appropriately expressed in the basal layer of the epidermis (Fig. 2.1A and Fig. 2.1C) as previously described (Greig et al. 2003d).

### **2.4.2. P2Y receptors are expressed in A375 human melanoma cells grown in culture**

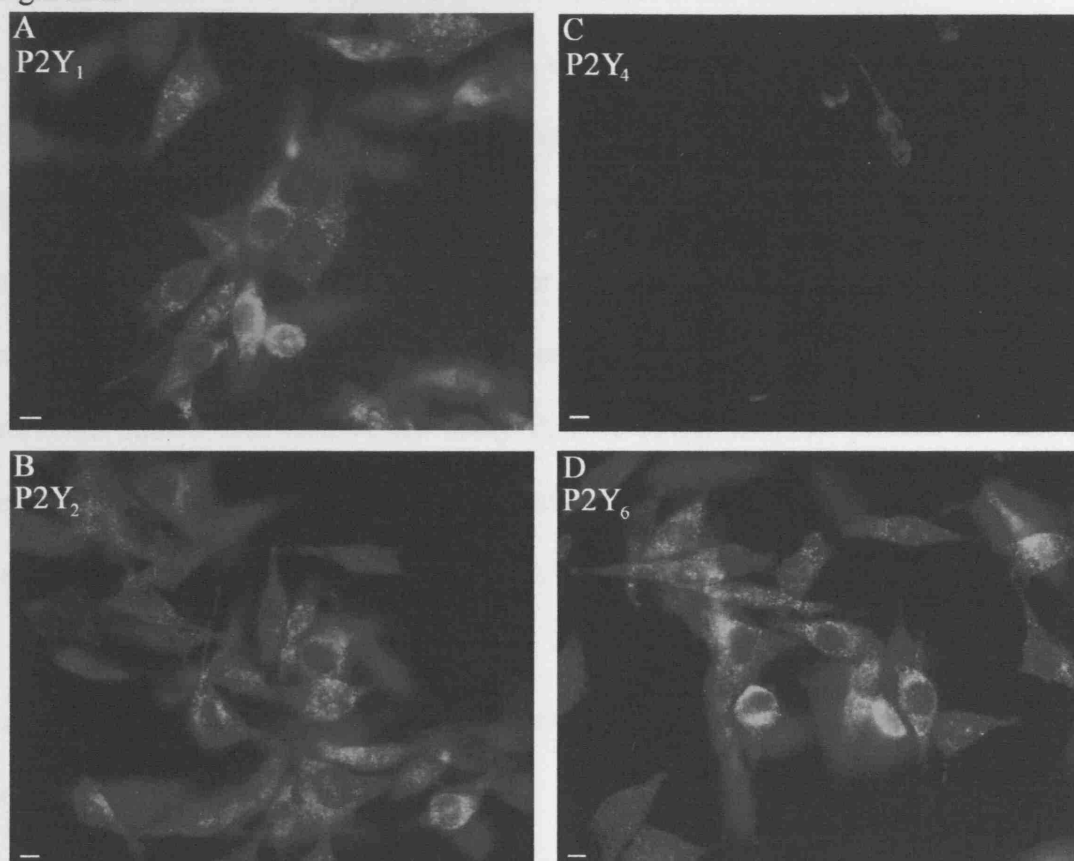
There was cell membrane expression of P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors by A375 melanoma cells grown in culture; no P2Y<sub>4</sub> receptors were seen (Fig. 2.2). In addition there was some punctuate immunoreactivity present inside the cells, this is the visualisation of endocytosis of P2Y receptors. Internalization of metabotropic P2Y receptors is a recognized and well described feature of these receptors (Tulapurkar et al. 2004). Both pre-absorption with the corresponding peptide and omission of the primary antibody were performed as controls which resulted in minimal immunoreaction.

Figure 2.1



P2Y receptors are expressed in formalin fixed, paraffin embedded tissue sections of melanoma. (A) Immunostaining of P2Y<sub>1</sub> receptors (pink) with haematoxylin counterstain (purple) in a specimen of melanoma. The melanoma (m) has infiltrated the dermis (d) and has nearly completely replaced it. Note that the staining of basal cells, indicated by arrows, in the stratum basale (sb) of the epidermis (e) in the same section is an internal positive control. Bar= 250  $\mu$ M. (B) Weak positive staining for P2Y<sub>2</sub> receptors of the core of the melanoma (m) with strong positive staining of cells at the proliferating margin (x) as it infiltrates the dermis (d). Bar= 500  $\mu$ M. (C) P2Y<sub>2</sub> receptor staining, indicated by arrows, are present both in melanoma cells (m) and consistently expressed by the basal cells of the epidermis (e) is an internal positive control. Bar= 500  $\mu$ M. (D) P2Y<sub>6</sub> receptors are consistently expressed by melanoma cells, throughout a tissue sample. Bar= 250  $\mu$ M.

Figure 2.2



Immunolocalisation of P2Y receptors in human melanoma cells grown in culture. Fluorescent staining was seen for P2Y<sub>1</sub> (A), P2Y<sub>2</sub> (B) and P2Y<sub>6</sub> (D) receptors, but P2Y<sub>4</sub> receptors were not present (C). Bar=25  $\mu$ M.

#### **2.4.3. mRNA for P2Y receptors is present in melanoma cells**

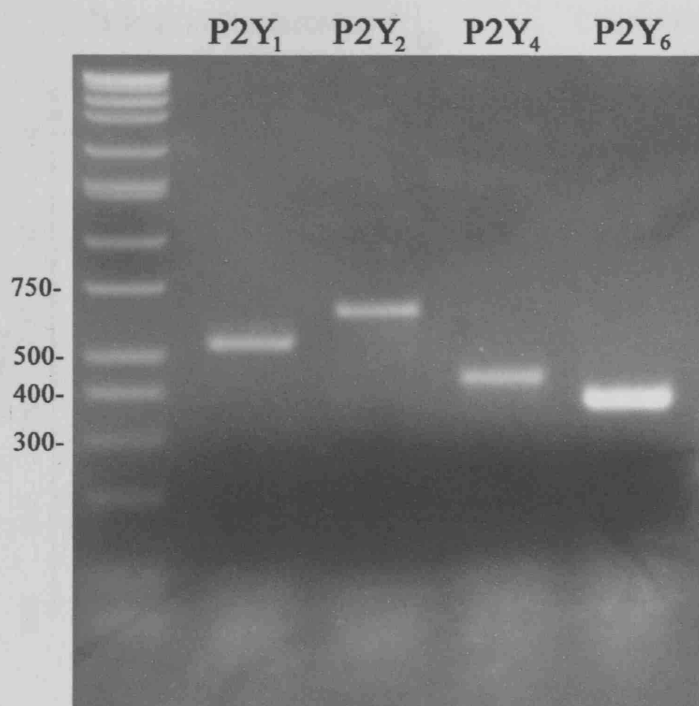
RT-PCR analysis of the total RNA extracted from the A375 melanoma cells showed a positive band at the expected sizes for all four P2Y receptors examined (Fig. 2.3). P2Y<sub>4</sub> mRNA was detected; however, since there was no specific immunostaining for P2Y<sub>4</sub> receptors in either cells or tissue, it is unlikely the mRNA is translated into P2Y<sub>4</sub> receptor protein, at least in the conditions examined. Previous work has shown that mRNA for many P2Y receptors may be present in various tissues and cells but this does not necessarily reflect functional receptor protein expression (Moore et al. 2001).

#### **2.4.4. Intracellular calcium levels are altered in response to purinergic compounds**

The concentration of intracellular calcium in the A375 melanoma cells was increased in a dose dependent manner by the addition of ATP (Fig. 2.4A). In a bath solution containing Ca<sup>2+</sup> there was a biphasic increase in intracellular calcium levels; an initial peak was followed by a second, sustained lower phase. When ATP was added to cells incubated in a calcium free bath solution, there was only the initial monophasic rise in intracellular calcium (Fig. 2.4B). The role of phospholipase-C coupled G-proteins in P2Y receptor mediated signalling (Burnstock 1997) was investigated using the phospholipase C specific inhibitor U73122. The ATP mediated rises in intracellular calcium were reduced by pre-incubation for 15 minutes with U73122 in a dose dependent manner, whilst the inactive analogue U73343 had minimal effect (Fig. 2.4C).

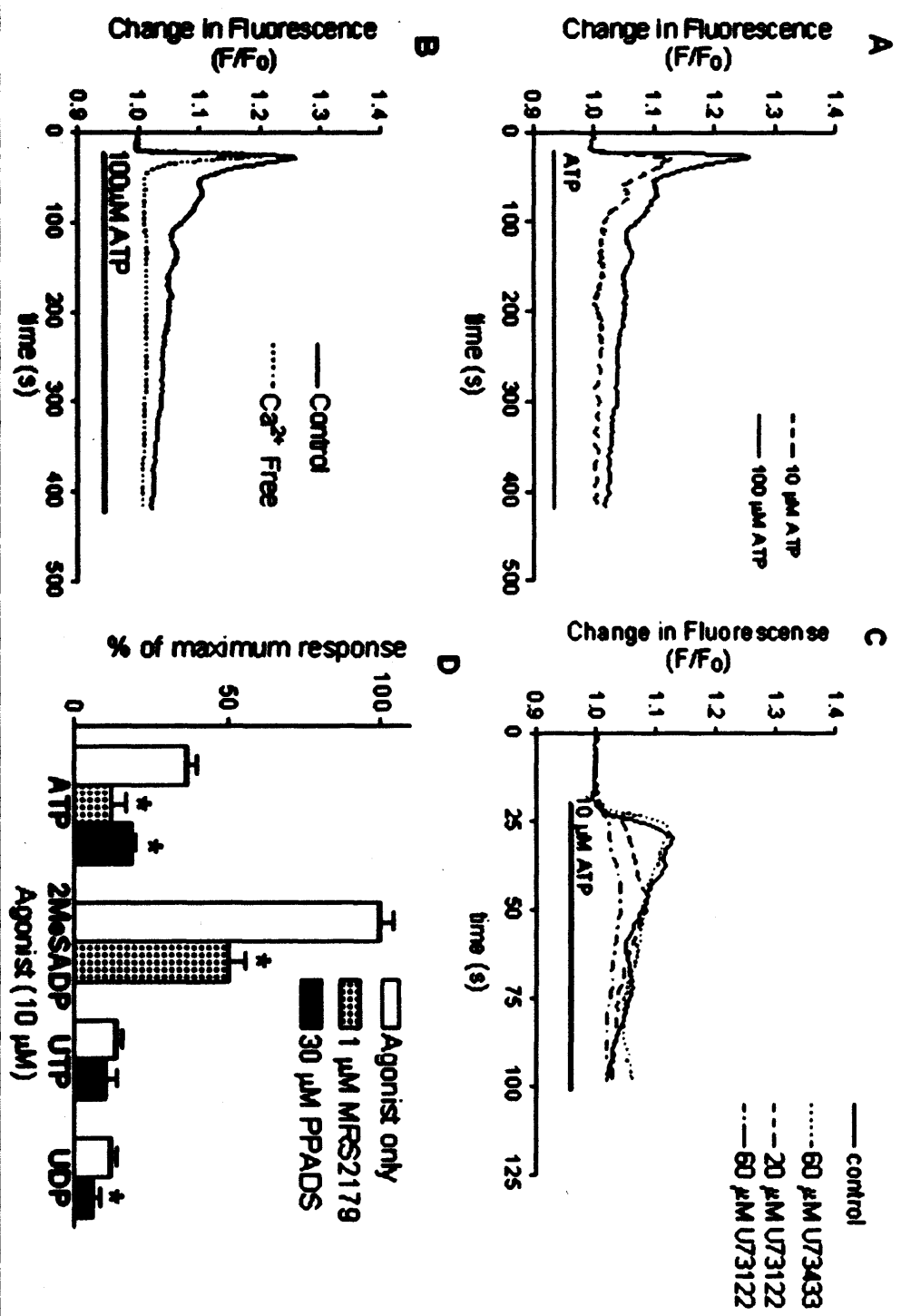
The biphasic response to stimulation by ATP is an established feature of metabotropic P2Y receptors (Maaser et al. 2002). The response to ATP seen is

Figure 2.3



mRNA for P2Y receptor subtypes P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub>, is present in melanoma cells. RT-PCR analysis of total RNA extracted from A375 cells generated fragments of the expected size.

Figure 2.4





#### Figure 2.4 Legend

Intracellular calcium levels, measured using the FLIPR, are raised in A375 melanoma cells after application of extracellular ATP. (A) Dose-dependent biphasic increase in intracellular calcium levels in a bath solution containing  $\text{Ca}^{2+}$ . (B) Rise in intracellular calcium level after application of ATP in a calcium free bath solution is monophasic. (C) Preincubation of cells with the phospholipase C inhibitor U73122 dose dependently reduced the rise in intracellular calcium in response to ATP. There was minimal change when U73122 was replaced by its inactive analogue U73343. Means of 3 independent experiments each consisting of 3 or more wells are shown. (D) P2Y receptor subtype agonists cause reversible increases in intracellular calcium levels. Application of different P2 agonists at equal concentration (10 $\mu\text{M}$ ) caused different increases in intracellular calcium. Values plotted are the peak response as a percentage of the maximum response of the agonist with the highest efficacy (2MeSADP). Cells were preincubated with the P2Y<sub>1</sub> receptor selective antagonist MRS 2179 and the P2Y<sub>1</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub> receptor selective antagonist PPADS for 30 minutes prior to the addition of agonist. Means  $\pm$  s.e.m. of 3 independent experiments each consisting of 3-8 wells are shown (\*  $p < 0.05$ ).

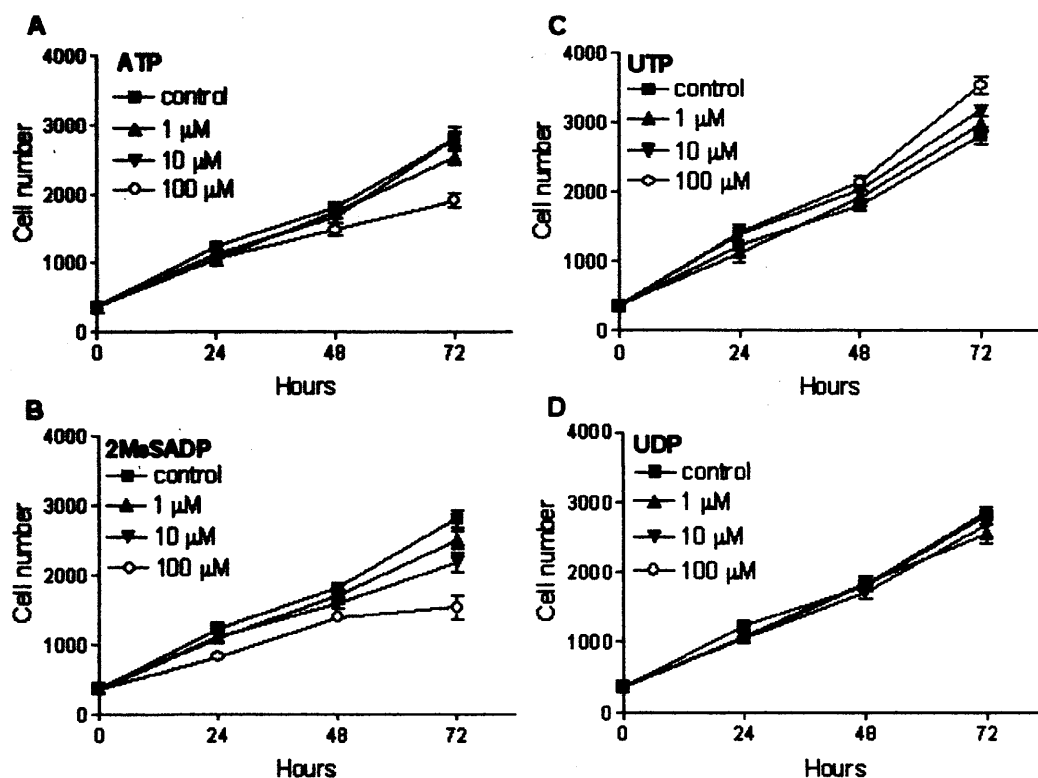
suggestive of an initial rise in intracellular calcium levels due to the mobilization of  $\text{Ca}^{2+}$  from intracellular stores by G-protein receptors coupled to the phospholipase C /  $\text{IP}_3$  transduction pathway. This is followed by a second peak in intracellular calcium levels due to calcium mediated calcium influx across the cell membrane.

To characterize the P2Y receptor subtypes present pharmacologically, changes in intracellular calcium levels were also measured in response to the P2Y<sub>1</sub> receptor-selective agonist 2MeSADP, the P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub> receptor agonist UTP and the P2Y<sub>6</sub> receptor selective agonist UDP. These compounds were used either alone or following preincubation of the cells with the P2Y<sub>1</sub> receptor selective antagonist MRS 2179 and the P2Y<sub>1</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub>, receptor antagonist PPADS. Each of the agonists produced an increase in intracellular calcium levels, which was partially reversed by preincubation with antagonist in all but one case. At a dose of 10  $\mu\text{M}$  the rank order of potency was 2MeSADP > ATP > UTP > UDP (Fig. 2.4D). The antagonist MRS 2179 caused a significant reduction in the response to ATP and 2MeSADP, whilst PPADS caused a significant reduction in the response to ATP and UDP and a minimal, non-significant reduction in the response to UTP. These results suggest the presence of functional P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>6</sub> receptor subtypes.

#### **2.4.5. Extracellular nucleotides regulate changes in A375 melanoma cell number**

The proliferative effects of a sustained application of the P2Y receptor agonists ATP, 2MeSADP, UTP and UDP was studied on human melanoma cells using a crystal violet colourimetric assay. The generic, nonspecific receptor subtype agonist ATP produced no change in cell number at low concentrations of 1  $\mu\text{M}$  and 10  $\mu\text{M}$  concentration and a decrease in cell number at a high concentration of 100  $\mu\text{M}$  (Fig. 2.5A). Seventy-two hours after treatment with 100  $\mu\text{M}$  ATP there was a

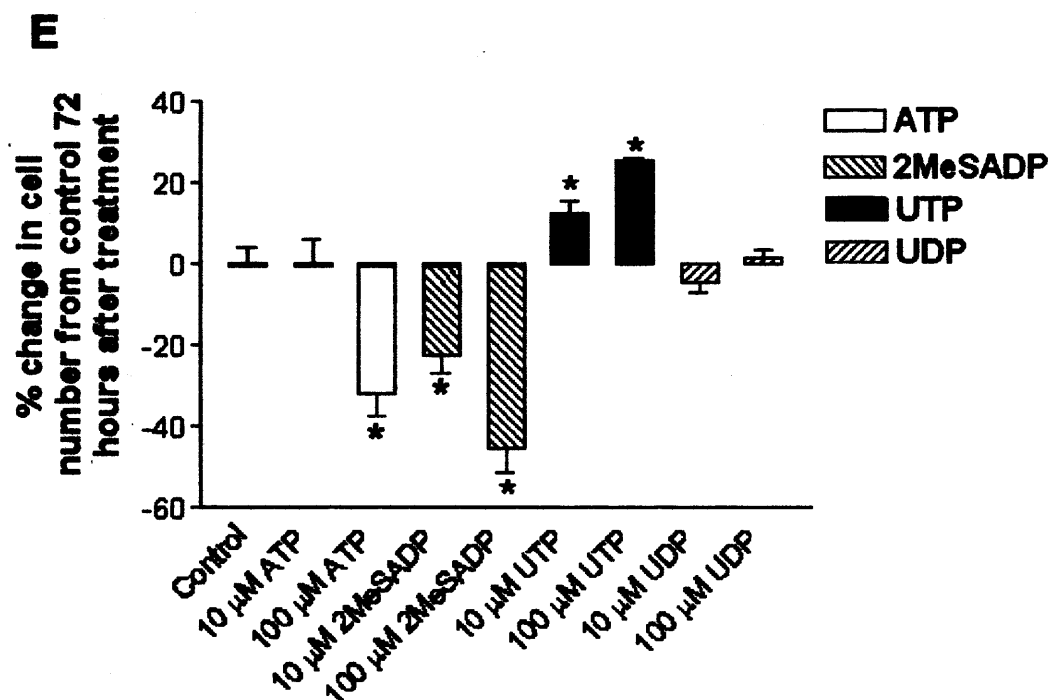
Figure 2.5A-D



Effect of extracellular nucleotides on melanoma cell proliferation. (A) ATP at a concentration of 100  $\mu$ M caused a significant reduction in cell number whereas 1  $\mu$ M and 10  $\mu$ M ATP had no effect. (B) 2MeSADP caused a dose dependent decrease in melanoma cell number. (C) UTP was shown to cause a dose dependent increase in cell number. (D) Treatment with UDP had no effect on cell number.

significant reduction of 32% in cell number compared to control ( $p < 0.05$ ). The P2Y<sub>1</sub> specific agonist 2MeSADP was shown to cause a dose dependent decrease in cell number compared to control (Fig 2.5B) with a 45% decrease in cell number 72 hours after treatment with 100  $\mu$ M 2MeSADP ( $p < 0.05$ ), whereas the P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub> receptor agonist UTP caused a dose dependent increase in cell number (Fig. 2.5C). There was an increase of 26% 72 hours after treatment with 100  $\mu$ M UTP ( $p < 0.05$ ). The selective P2Y<sub>6</sub> receptor agonist UDP showed no effect on changes in cell number (Fig. 2.5D). These results are summarised in Fig. 2.5E.

Figure 2.5E



Effect of extracellular nucleotides on melanoma cell proliferation. (E) Summary of results 72 hours after treatment with purinergic agonist. Means  $\pm$  s.e.m. of a minimum of 3 different experiments each of a minimum of 3 wells are shown ( $n=9$  or greater), \* $p<0.05$ .

## 2.5. Discussion

ATP is known to inhibit cancer cell growth in a variety of models and through a number of mechanisms (Abraham et al. 2003). Attempts have been made to identify the mechanism of action of ATP on cancer cells and the purinergic receptor subtypes involved. Alteration of cancer cell number may be due to mediation of cell proliferation via P2Y receptors (Dubyak and De Young 1985; Lin and Chuang 1993; Maaser et al. 2002), stimulation of differentiation with subsequent inhibition of proliferation via P2X<sub>5</sub> receptors (Cowen et al. 1991; Popper and Batra 1993; Ryten et al. 2002) or induction of cell death (apoptosis) via P2X<sub>7</sub> receptors (Chueh and Kao 1993; Gartland et al. 2001). P2Y receptors have been described in cancer types, other than melanoma, where they mediate changes in cell number. However P2Y receptors may mediate opposite actions in different cancer types. For instance, P2Y<sub>2</sub> receptors have been shown to mediate an increase in cell number in lung cancer (Schafer et al. 2003) and squamous cell carcinoma of the skin (Greig et al. 2003c), but cause a decrease in proliferation in endometrial (Katzur et al. 1999) and colorectal (Hopfner et al. 1998) cell lines.

In this paper we provide evidence for functional P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors in the human melanoma A375 cell line at both the cellular and molecular levels and that activation of the P2Y<sub>1</sub> receptor causes a decrease in cell number whereas activation of the P2Y<sub>2</sub> receptor causes an increase in cell number.

The expression of both P2Y receptor mRNA and protein in the human A375 melanoma cell line was consistent with P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors being elucidated by both techniques. P2Y<sub>4</sub> receptor mRNA was expressed, but it was not translated into P2Y<sub>4</sub> receptor protein. This pattern of expression was also seen in formalin-fixed, paraffin-embedded specimens of melanoma which had been excised

from patients. P2Y<sub>1</sub> and P2Y<sub>6</sub> receptors were consistently expressed in these specimens. P2Y<sub>2</sub> receptors were also present but were expressed most strongly at the growing edges of the tumour specimens; this is consistent with mediating the proliferation of cells.

The dose dependent increases in intracellular calcium levels in response to ATP, which was biphasic in a Ca<sup>2+</sup>-containing bath solution but monophasic in a Ca<sup>2+</sup>-free bath solution and inhibited in a dose dependent manner by U73122 strongly suggests that the action of extracellular nucleotides is mediated by metabotropic P2Y receptors coupled to phospholipase C (Maaser et al. 2002).

Currently no selective agonists exist for all the purinoreceptors (Ralevic and Burnstock 1998) and therefore pharmacological identification relies on the effects of a combination of compounds. ATP itself is active over the entire range of P2Y receptors, although it has different efficacies at each receptor. 2MeSADP is a selective agonist at P2Y<sub>1</sub> receptors, UTP is an agonist at P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub> receptors and UDP is a selective agonist at the P2Y<sub>6</sub> receptor. MRS 2179 is a selective antagonist at the P2Y<sub>1</sub> receptor whereas PPADS is an antagonist at the P2Y<sub>1</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub> receptors but not at the P2Y<sub>2</sub> receptor.

2MeSADP caused an increase in intracellular calcium which was partially antagonised by MRS 2179; this is strong evidence for the presence of functioning P2Y<sub>1</sub> receptors. There was also an increase in intracellular calcium in response to UDP which was partially blocked by PPADS; again this is evidence for the presence of functioning P2Y<sub>6</sub> receptors, although little effect on cell proliferation was seen. UTP, active at P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub> receptors, also caused a response. As this was not reduced by the antagonist PPADS, an antagonist at P2Y<sub>1</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub>

receptors, it is likely the response to UTP is at least partially mediated via the P2Y<sub>2</sub> receptor.

The effect of incubation of A375 melanoma cells in the presence of 2MeSADP was a statistically significant dose dependent decrease in cell number. A reduction in cell proliferation mediated by the P2Y<sub>1</sub> receptor has previously been reported in human astrocytoma cancer cells (Sellers et al. 2001). UTP caused a dose dependent increase in cell number; the data from the RT-PCR, immunocytochemistry and intracellular calcium studies suggests this increase was mediated by P2Y<sub>2</sub> receptors. No change in cell number was seen when cells were incubated with UDP. ATP has an approximately equal affinity at the P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors, so even though no change in cell number was seen when 1-10 $\mu$ M ATP was applied, it is possible that ATP is having equal and opposite effects on cell number, mediated through both P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors. At a higher dose of 100  $\mu$ M there was a decrease in cell number though this was not as great as the decrease seen with 2MeSADP, so other receptor subtypes causing an increase in cell number such as the P2Y<sub>2</sub> might also still be activated.

Four of the currently described P2Y receptors have been studied in detail in this paper. As more P2Y receptor subtypes are described and tools for their study, such as specific antibodies, become available it is possible that other P2Y receptors may be shown to have a role in regulation of melanomas which may be linked to other intracellular messenger systems. This is suggested by the antagonists PPADS, MRS 2179 and U73122 incompletely blocking the effect of the purinergic agonists used.

In summary, we have shown for the first time, there are functional P2Y receptors in human melanomas which regulate cell proliferation. P2Y<sub>1</sub> receptors cause a decrease in cell number whereas P2Y<sub>2</sub> receptors cause an increase in cell



number. Functional P2Y<sub>6</sub> receptors are also present but were shown not to have an effect on cell number, suggesting they may play another role in cell regulation. Targeting purinergic metabotropic receptors may be a putative treatment for malignant melanoma.

## **Chapter Three**

### **Human melanomas express functional P2X<sub>7</sub> receptors**

### **3.1. Abstract**

Adenosine 5'-triphosphate is known to function as a potent extracellular messenger producing its effects via a distinct family of cell surface receptors. Different receptor subtypes have been shown to modulate different cellular functions such as proliferation, differentiation and apoptosis. We investigated the functional expression and apoptotic action of the P2X<sub>7</sub> receptor in human malignant melanoma tissue and cells. Incubation of cells with the potent P2X<sub>7</sub> receptor agonist 2'-3' -O-(4-benzoyl-benzoyl) adenosine 5'-triphosphate (BzATP) led to a decrease in cell number which was dose-dependent and reversible by the antagonist 1-N,O-bis-[5-isoquinoline-sulfonyl]-N-methyl-L-tyrosyl)-4-phenyl-piperazine (KN-62). Synthesis of the P2X<sub>7</sub> receptor was established by reverse transcriptase-polymerase chain reaction, immunohistochemistry, immunocytochemistry and cellular accumulation of the fluorescent, DNA binding dye YO-PRO-1. P2X<sub>7</sub> receptors were shown to mediate apoptotic actions of extracellular nucleotides and are a novel target for melanoma therapy.

### 3.2. Introduction

Malignant melanoma is an aggressive cancer that originates from melanocytes, the pigment-producing cells of the skin. The incidence of melanoma is increasing and the outcome for patients with advanced disease remains poor (Dreiling et al. 1996). This is due to a high level of therapeutic resistance (Serrone and Hersey 1999), therefore new treatment strategies are needed.

In addition to its key role in cellular metabolism, where it acts as a ubiquitous enzyme co-factor and as the key source of the cellular energy unique to phosphate bond formation, the purine nucleotide adenosine 5'-triphosphate (ATP) also functions as a potent extracellular messenger, producing its effects via a distinct family of cell surface receptors (Ralevic and Burnstock 1998). ATP was first shown to be a co-transmitter with noradrenaline in sympathetic nerves in smooth muscle cells of the vas deferens (Sneddon and Burnstock 1984). Sympathetic co-transmission has also been clearly demonstrated in a variety of blood vessels (Burnstock 1988). Co-transmission with acetylcholine in parasympathetic nerves has also been proposed in organs such as the urinary bladder (Burnstock et al. 1972; Hoyle and Burnstock 1993).

There exists a large family of extracellular receptors for ATP and related molecules. Purinergic receptors are divided into two groups: P1 purinoceptors are selective for adenosine and P2 purinoceptors are selective for ATP. Four different P1 adenosine receptors have been cloned, which are coupled to G proteins (Fredholm et al. 2001). These receptors are widely distributed in the human body regulating the function of virtually every organ and tissue. Two families of P2 purinoceptors are currently recognised: the P2X gated ion channel and the P2Y G protein-coupled receptor family. Seven subtypes of P2X receptors have been described and eight subtypes of P2Y receptors (Abbracchio and Burnstock 1994). Different receptors

have been shown to have different functions. For instance the P2Y<sub>2</sub> receptor plays a role in cellular proliferation (Greig et al. 2003d), the P2X<sub>5</sub> receptor is implicated in differentiation (Ryten et al. 2002) and activation of the P2X<sub>7</sub> receptor leads to apoptosis (Humphreys et al. 2000).

The P2X<sub>7</sub> receptor was first cloned from rat brain and from macrophages in 1996 (Surprenant et al. 1996). P2X<sub>7</sub> receptors are formed by the aggregation of homomeric P2X<sub>7</sub> receptor subunits (Torres et al. 1999); which combine to form a ligand-gated ion channel which allows passage of cations when activated. Each subunit consists of two transmembrane domains, separated by an extensive N-glycosylated extracellular loop, always containing ten cysteine residues, and intracellular amino (N) and carboxy (C) termini.

P2X<sub>7</sub> receptors are non-selective cation channels. Under normal physiological conditions, P2X<sub>7</sub> receptor activation will result in to Na<sup>+</sup> and Ca<sup>2+</sup> influx and K<sup>+</sup> efflux across the cell membrane leading to depolarisation of the plasma membrane and an increase in intracellular to Na<sup>+</sup> and Ca<sup>2+</sup> concentration. Membrane depolarisation can in turn activate voltage-gated channels, causing firing of action potentials. The P2X<sub>7</sub> receptor functions like other P2X receptors when briefly activated, but when exposed to at high concentration or for a long period to ligands such as ATP the cation channel can also be converted to a large non-selective transmembrane pore which allows the passage of not only cations but small molecules up to the size of 900 daltons (Surprenant et al. 1996). This effect is associated with mediating a number of physiological processes including apoptosis mediated through the caspase enzyme system (Humphreys et al. 2000), release of cytokines such as interleukin-1 $\beta$ , interferon- $\gamma$  and tumour necrosis factor- $\alpha$  (Ferrari et al. 2000;Humphreys and Dubyak 1996;Mehta et al. 2001), vesicle release (MacKenzie

et al. 2001) and cell fusion (Chiozzi et al. 1997). P2X<sub>7</sub> receptors have been described in a wide range of human tissues and cells including lymphocytes (Wiley et al. 1998), macrophages (Chiozzi et al. 1997), dendritic cells (Coutinho-Silva et al. 1999) and keratinocytes (Greig et al. 2003d).

Raised extracellular ATP has been shown to inhibit tumour growth *in vitro* and *in vivo* (Rapaport 1983b; Rapaport and Fontaine 1989d). There have been several clinical trials for the beneficial use of ATP against cancer (Agteresch et al. 2000; Haskell et al. 1996). Attempts have been made to identify the mechanism of action of ATP on cancer cells and the purinergic receptor subtypes involved. A decrease in cancer cell number may be due to an inhibition of cell proliferation mediated via P2Y receptors (Dubyak and De Young 1985; Lin and Chuang 1993; Maaser et al. 2002), stimulation of differentiation with subsequent inhibition of proliferation via P2X<sub>5</sub> receptors (Cowen et al. 1991; Popper and Batra 1993) or induction of cell death (apoptosis) via P2X<sub>7</sub> receptors (Chueh and Kao 1993).

P1 receptors have been described in the human melanoma A375 cell line (Merighi et al. 2001), with evidence for all four subtypes of receptor being present and it has been postulated that they may be a target for treatment of this cancer (Merighi et al. 2002). Recently, evidence for the presence of P2X<sub>7</sub> receptors in human melanomas was demonstrated using immunohistochemistry on specimens of superficial spreading melanoma (Slater et al. 2003a).

In this study, we confirm the presence of P2X<sub>7</sub> receptors in human melanoma tissue and show that the A375 melanoma cell line expresses functional P2X<sub>7</sub> receptors at the cellular and molecular level and activation of this receptor causes apoptosis of melanoma cells in a dose-dependent manner.

### **3.3. Materials and methods**

#### **3.3.1. Cell culture**

The melanoma cell line A375 (Giard et al. 1973b) was obtained from the Wellcome Trust Functional Genomics Cell Bank (St Georges Hospital Medical School, London, UK). Melanoma cells were grown in 90% Dulbecco's modified Eagle's medium (DMEM) and 10% heat inactivated fetal calf serum supplemented with penicillin (100 U per ml), streptomycin (100 µg per ml) and L-glutamine (2 mM) in 75 cm<sup>2</sup> tissue culture flasks (Corning, New York, USA). All other cell culture reagents were obtained from Sigma (Poole, UK). Cells were incubated at 37°C in 5% CO<sub>2</sub> /95% air and were sub-cultured at 70% confluence. Cell viability was determined using the trypan blue exclusion method.

#### **3.3.2 Reverse transcriptase polymerase chain reaction**

The synthesis of mRNA encoding the seven human P2X receptor subtypes in A375 melanoma cells was determined using reverse transcriptase (RT) and the polymerase chain reaction (PCR). Total RNA was isolated from A375 cells grown in culture, to confluence, using the SV Total RNA Isolation System (Promega, WI, USA). The RT-PCR reactions were carried out using Amersham Biosciences Ready-To-Go RT-PCR beads (New Jersey, USA). RT was carried out on 1 µg of RNA for 30 minutes at 42°C. The PCR cDNA samples were initially denatured for 5 minutes at 95°C prior to the addition of the primer sequences. The human P2X primer sequences used were: P2X<sub>1</sub> 5'-gac aac tcc ttc gtg gtc at-3' and 5'-ccg tac gtg cca gtc cag gt-3' product size 510 base pairs; P2X<sub>2</sub> 5'-gca tcg gag tgc aac ccc aa-3' and 5'-tca cag gcc agc tac ct gag-3' product size 355 base pairs; P2X<sub>3</sub> 5'atc aac cga gta gtt cag c-3' and 5'-gat gca ctg gtc cca gg-3' product size 695 base pairs; P2X<sub>4</sub> 5'-gag att cca gat gcg

acc-3' and 5'-gac ttg agg taa gta gtg g-3' product size 295 base pairs; P2X<sub>5</sub> 5'-tcg act aca aga cc gaga ag-3' and 5'-ctt gac gtc cat cac att g-3' product size 595 base pairs; P2X<sub>6</sub> 5'-aaa aac agg cca gtg tgt ggt gtt c-3' and 5'-tgc ctg ccc ggt gac gag gat gtc ga-3' product size 520 base pairs; P2X<sub>7</sub> 5'-aac atc act tgt acc ttc c-3' and 5'-tgt gaa gtc cat cgc agg-3' product size 674 base pairs (Adrian et al. 2000; Janssens and Boeynaems 2001; Nakamura et al. 2000) and were obtained from Invitrogen, Paisley, UK. The PCR cDNA samples were run for 35 cycles (30 s at 95 °C, 1 minute at 54-60 °C and 1 minute at 72 °C) with a final extension at 72 °C for 5 minutes in a DNA thermal cycler (Hybaid, UK). The size of the PCR products was determined using agarose gel electrophoresis (Gibco, Paisley, UK) and compared with a DNA ladder (Sigma, Poole, UK).

### **3.3.3. Immunohistochemistry of paraffin-embedded specimens**

A total of 14 specimens of melanoma of differing histological subtypes were examined. Paraffin blocks were sectioned at 4 µm on a Reichert-Jung Microtome, and sections were taken on Snowcoat Extra slides (Surgipath, Cambridgeshire, UK), then dried in an oven for 2 hours at 60°C. Sections were dewaxed and rehydrated using xylene and graded concentrations of ethanol. Antigen retrieval was performed by microwaving for 10 minutes in a solution of 1 mM ethylenediamine tetraacetic acid (Tris EDTA) at pH 9.0. Endogenous alkaline phosphatase was blocked by a 20 minute incubation in 20% acetic acid. Sections were washed and then incubated with avidin D blocking solution, biotin blocking solution and 1:5 normal swine serum (Vector laboratories).

100 µl of P2X<sub>7</sub> receptor antibody, diluted 1:100, was applied for 12 hours at 4°C. The immunogen used for production of the polyclonal P2X<sub>7</sub> antibody was a



synthetic peptide corresponding to 15 receptor-type-specific amino acids (peptide sequence TWRFGSQDMADFAIL) in the intracellular C-termini of the cloned human P2X<sub>7</sub> receptor, in a similar method to that previously described (Oglesby, 1999 ). The polyclonal antibody was raised by multiple monthly injections of New Zealand rabbits with the peptide.

100 µl of biotinylated anti-rabbit antibody (DAKO E0353), diluted 1:200 in DAKO ChemMate diluent was applied for 30 minutes followed by 100 µl of streptavidin alkaline phosphatase (Vector SA5100) diluted 1:200 in DAKO ChemMate for 30 minutes. Vector Red substrate (Vector alkaline phosphatase substrate, SK5100) prepared in 200mM Tris-HCl (pH 8.2) was then applied for 10 minutes. Positive P2X<sub>7</sub> staining appeared bright pink, nuclei were counterstained purple with hematoxylin. All sections were subsequently dehydrated, cleared and mounted. Negative controls were performed by either omission of the primary antibody or preabsorption of the primary antibody with the corresponding peptide sequence.

#### **3.3.4. Immunocytochemistry of cells grown in culture**

A375 cells were grown in culture on chamber slides (Nunc, Illionois, USA). They were fixed in 4% formaldehyde in 0.1 M phosphate buffer for 2 minutes. Non-specific binding sites were blocked by a 20 minute preincubation with 10% normal horse serum (NHS) in 0.1 M phosphate buffer containing 0.05% merthiolate, followed by incubation with primary P2X<sub>7</sub> antibody diluted 1:100, with 0.2% Triton x-100, for 12 hours at 4°C. Subsequently, the slides were incubated with donkey anti-rabbit Cy3 (Jackson ImmunoResearch, Pennsylvania, USA) diluted 1:300 with 1% NHS in phosphate buffer. Slides were then mounted with Eukitt (BDH laboratories, Dorset,

UK) and examined. Control experiments were carried out with the primary antibody being omitted from the staining procedure or the primary antibody preabsorbed with the corresponding peptide. All other reagents were obtained from Sigma (Poole, UK).

### **3.3.5. YO-PRO-1 fluorescent imaging of P2X<sub>7</sub> receptor mediated pore formation**

A375 human melanoma cells were grown in 96-well plates (BD Falcon, New Jersey, USA) to confluence. For the measurement of P2X<sub>7</sub> receptor mediated pore formation cells were incubated in an extracellular bath solution of: 20 mM HEPES, 140 mM NaCl, 4 mM KCl, 1 mM K<sub>2</sub>HPO<sub>4</sub>, 1 mM CaCl<sub>2</sub> and 10 mM D-Glucose with the pH adjusted to 7.4 with 1 M NaOH; containing 1  $\mu$ M YO-PRO-1 (Molecular Probes, Eugene, OR, USA). The plates were placed into a Fluorometric Imaging Plate Reader (FLIPR- Molecular Devices, Ca, USA), where they were incubated at 37°C, prior to preincubation with the P2X<sub>7</sub> selective antagonist 1-N,O-bis-[5-isoquinoline-sulfonyl]-N-methyl-L-tyrosyl)-4-phenyl-piperazine (KN-62) for 20 minutes and the addition of the P2X<sub>7</sub> selective agonist 2'-3' -O-(4-benzoyl-benzoyl) adenosine 5'-triphosphate (BzATP). Both compounds were obtained from Sigma (Poole, UK). The cells were excited by an argon-ion laser source at a wavelength of 488 nm and emission was measured at 520 nm at 30 second intervals for 60 minutes. The change in fluorescence measured was plotted in arbitrary units as a change from baseline fluorescence prior to the addition of BzATP.

### **3.3.6. Cell proliferation assay**

A375 human melanoma cells were seeded onto a 96-well plate at a density of 250 cells per well (2,500 cells per ml with 100  $\mu$ l of cell suspension per well). This seeding density gave the best growth curve over a 96 hour period. 24 hours after

seeding, the medium was aspirated and fresh medium containing P2X<sub>7</sub> receptor agonist BzATP and/or P2X<sub>7</sub> antagonist antagonist KN-62, or just medium (control) was added to the well plates. Prior to addition to the cells, the pH of the drug solutions prepared in the culture medium was adjusted to 7.36-7.44 with 1 M NaOH.

Changes in cell number were quantified via a colorimetric assay using crystal violet (Gillies, 1986) and read using a spectrophotometric plate reader (Labtech, East Sussex, UK) at 48 hours after addition of the purinergic agents.

For the colorimetric assay, a solution of 0.5 g crystal violet, 0.85 g NaCl, 5 ml 10% formal saline, 50 ml absolute ethanol, 45 ml distilled water was used. Medium was aspirated from the wells and 100 µl of the colorimetric assay mixture was added to each well and incubated at room temperature for 10 minutes. This mixture allowed simultaneous fixation of cells and penetration of the crystal violet dye into the cells. After washing 3 times with phosphate buffered saline (PBS), 33% acetic acid was used to elute colour from the cells and the optical density was read at a wavelength of 570 nm using a spectrophotometric plate reader (Labtech, East Sussex, UK). To confirm that the optical density of the wells correlated with cell number, a control assay was performed for each experiment where known numbers of cells were seeded in ascending seeding densities and the plate read as soon as the cells had attached. Cell number versus optical density was plotted. The R<sup>2</sup> value of the trend line was always greater than 0.98.

### **3.3.7. Caspase 3/7 apoptosis assay**

A375 melanoma cells were incubated with 100 µl medium containing 30 µM to 300 µM BzATP for 6 hours in a 96-well white-walled luminometer plate. 100 µl of Caspase-Glo 3/7 reagent (Promega, WI, USA) was added to each well to be

measured, including blank baseline and untreated control wells. The contents were gently mixed at 300 rpm for 30 seconds and then incubated at room temperature for 1 hour. Luminescence was then measured with a plate reading luminometer (Labtech, East Sussex, UK). The blank base line signal was subtracted from the signal produced by the treated and untreated cells.

### **3.4. Results**

#### **3.4.1. P2X<sub>7</sub> receptor mRNA is present in A375 melanoma cells**

RT-PCR analysis of P2X receptor subtypes demonstrated a positive band at the expected size for the P2X<sub>7</sub> receptor (Fig. 3.1). No mRNA for the P2X<sub>1-6</sub> receptor subtypes was detected.

#### **3.4.2. P2X<sub>7</sub> receptors are present in paraffin-embedded sections of melanoma**

Positive labelling for P2X<sub>7</sub> receptors was found to be present in all specimens of melanoma examined (Fig. 3.2a). Over 50% of the cells in each specimen were positive for P2X<sub>7</sub> receptors and in the majority of specimens over 75% of the cells stained positive. There was no difference between different histological subtypes in the number of cells in each specimen with positive staining for P2X<sub>7</sub> receptors. As an internal positive control, P2X<sub>7</sub> receptors were also appropriately stained in the stratum corneum of the epidermis (Fig. 3.2b) as previously described (Greig, 2003).

#### **3.4.3. P2X<sub>7</sub> receptors are expressed in A375 human melanoma cells grown in culture**

There was synthesis of P2X<sub>7</sub> receptors by A375 melanoma cells grown in culture (Fig. 3.3). Both pre-absorption with the corresponding peptide and omission of the primary antibody were performed as controls. There was minimal immunoreaction with preabsorption of the primary antibody and when the primary antibody was omitted.

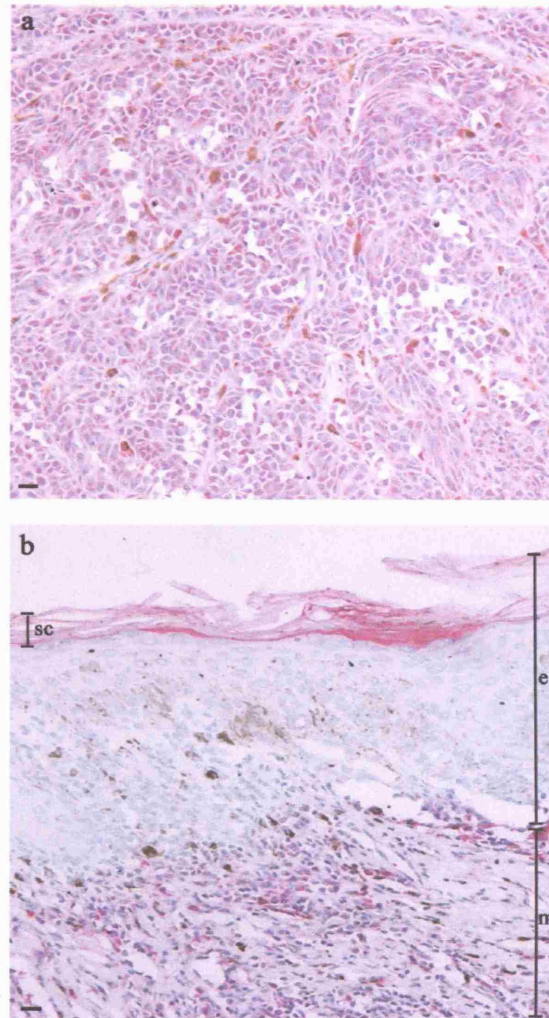
Figure 3.1

**P2X<sub>1</sub> P2X<sub>2</sub> P2X<sub>3</sub> P2X<sub>4</sub> P2X<sub>5</sub> P2X<sub>6</sub> P2X<sub>7</sub>**

**674 bp**

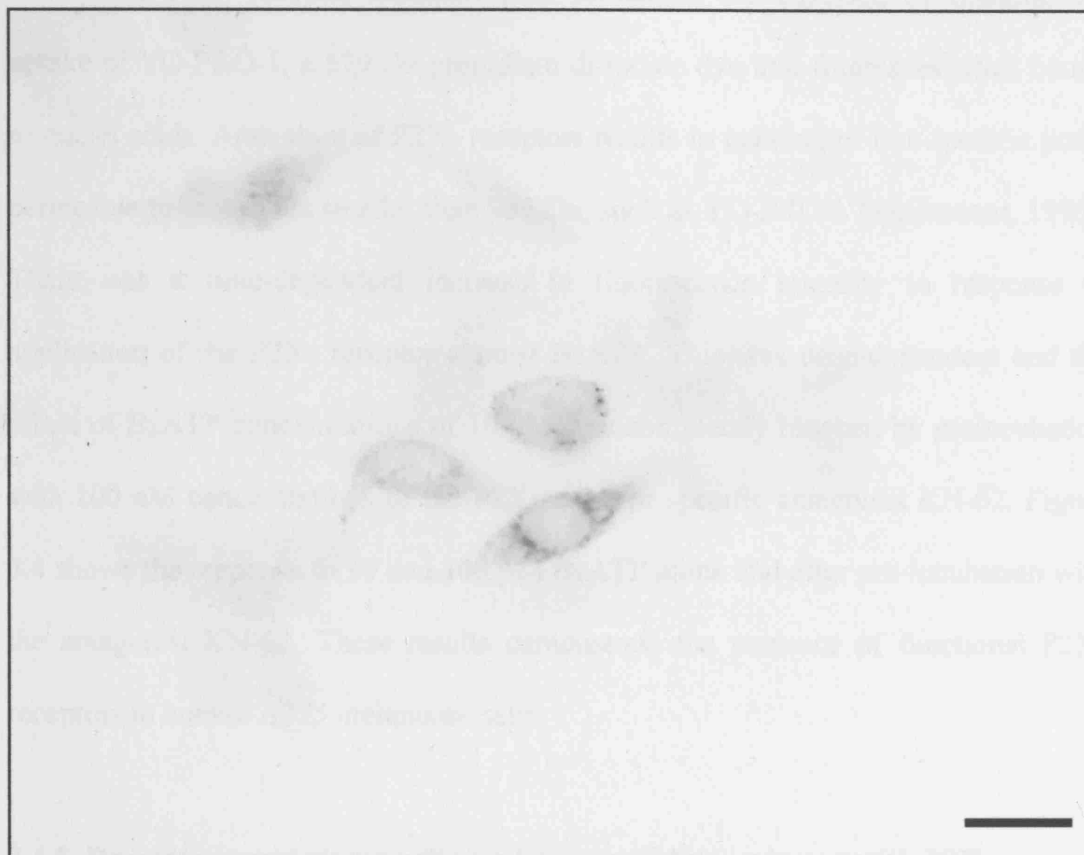
P2X<sub>7</sub> receptor mRNA is present in melanoma cells. RT-PCR analysis of total RNA extracted from A375 melanoma cells generated a fragment of the expected size of 674 base pairs for the P2X<sub>7</sub> receptor. mRNA for no other P2X receptor subtypes was demonstrated.

Figure 3.2



P2X<sub>7</sub> receptors are present in sections of melanoma. (a) Streptavidin alkaline phosphatase immunostaining of P2X<sub>7</sub> receptors (pink) with haematoxylin counterstain (purple) in a formalin-fixed, paraffin-embedded melanoma. Bar 250  $\mu$ m. (b) Staining of both melanoma cells infiltrating the dermis [m] and the stratum corneum [sc] of the epidermis in the same section is an internal positive control. Note no positive staining in the other layers of the epidermis [e]. Bar 500  $\mu$ m.

Figure 3.3



P2X<sub>7</sub> receptors are present in A375 human melanoma cells grown in culture. P2X<sub>7</sub> receptor fluorescent extracellular staining in human melanoma A375 cells grown in culture. Bar 25 μM.



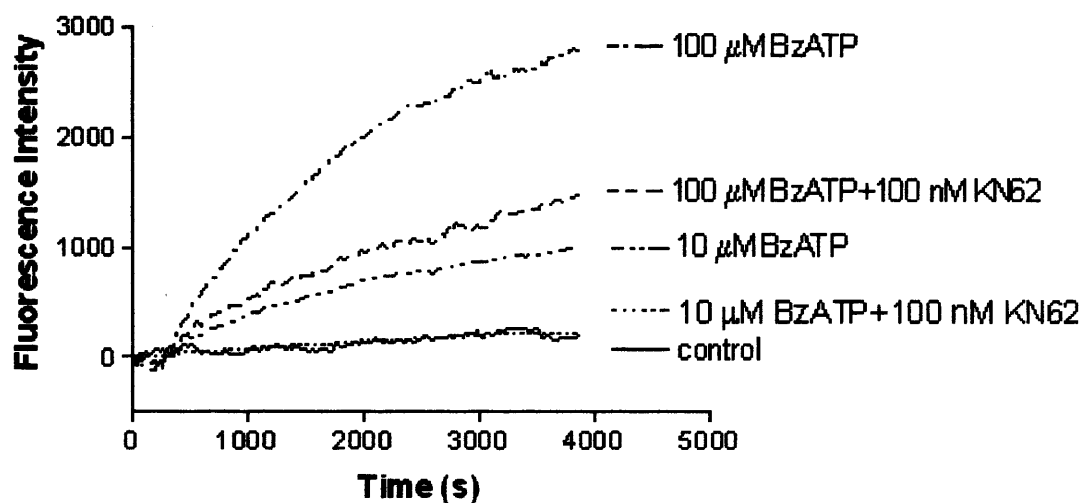
#### **3.4.4. P2X<sub>7</sub> receptor agonist mediates YO-PRO-1 uptake**

The P2X<sub>7</sub> receptor mediated pore formation was assessed by imaging the uptake of YO-PRO-1, a 629 Da propidium di-iodide dye that fluoresces when bound to nuclei acids. Activation of P2X<sub>7</sub> receptors results in opening of non-specific pores permeable to molecules smaller than 900 Da, such as YO-PRO-1 (Surprenant, 1996). There was a time-dependent increase in fluorescence intensity in response to application of the P2X<sub>7</sub> receptor agonist BzATP. This was dose-dependent and the effect of BzATP concentrations of 10  $\mu$ M was completely blocked by preincubation with 100 nM concentrations of the P2X<sub>7</sub> receptor specific antagonist KN-62. Figure 3.4 shows the response to 10 and 100  $\mu$ M BzATP alone and after pre-incubation with the antagonist KN-62. These results demonstrate the presence of functional P2X<sub>7</sub> receptors in human A375 melanoma cells.

#### **3.4.5. Decrease in melanoma cell number caused by treatment with P2X<sub>7</sub> receptor agonist is reversed by competitive antagonist**

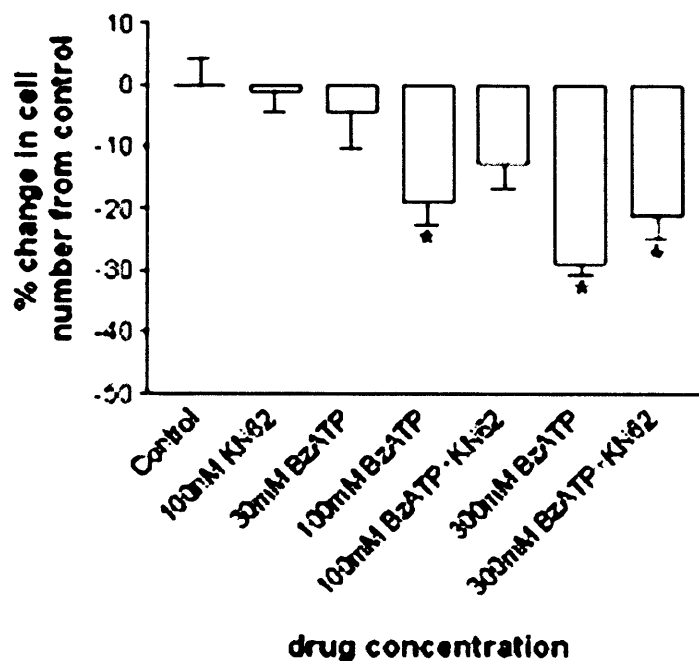
The proliferative effects of a sustained application of the P2X<sub>7</sub> receptor agonist BzATP was studied on human melanoma cells using a crystal violet colorimetric assay. BzATP was found to cause a dose-dependent decrease in cell number which was partially reversed by addition of the competitive antagonist KN-62 (Fig. 3.5). At a dose of 100  $\mu$ M BzATP, or higher, a significant ( $p < 0.001$ ) reduction in cell number was seen after 48 hours. A maximum decrease in cell number of 29% was obtained with 300  $\mu$ M BzATP. This decrease in cell number was partially reversed in the presence of 100 nM KN-62, with only a 21% decrease being measured when treated with 300  $\mu$ M BzATP. 100 nM KN-62 also partially reversed the decrease in cell number caused by 100  $\mu$ M BzATP, but this was not statistically

Figure 3.4



Demonstration of functional P2X<sub>7</sub> receptors in human A375 melanoma cells. The P2X<sub>7</sub> receptor agonist BzATP mediated uptake of the fluorescent, DNA binding dye YO-PRO-1. There was a time-dependent increase in fluorescence intensity in response to application of the P2X<sub>7</sub> receptor agonist BzATP at timepoint 300s. This was dose-dependent and the effect of BzATP concentrations of 10  $\mu$ M was completely blocked by preincubation with 100 nM concentrations of the P2X<sub>7</sub> receptor specific antagonist KN-62.

Figure 3.5



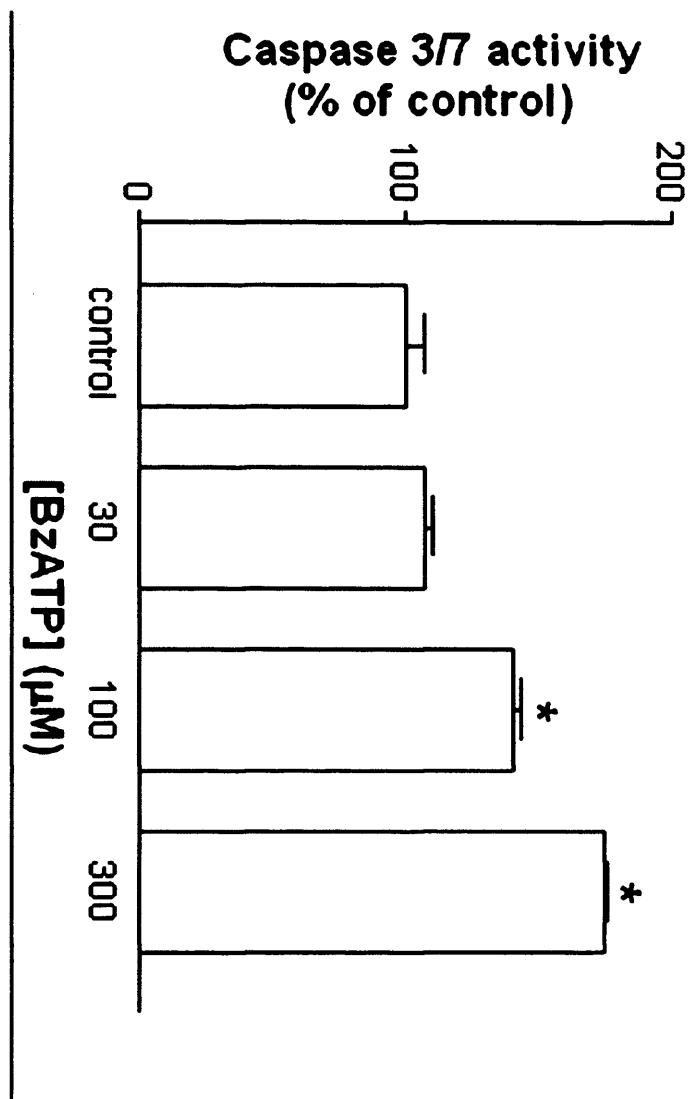
Cell proliferation assay. The proliferative effects of a sustained application of the P2X<sub>7</sub> receptor agonist BzATP was studied on human melanoma cells using a crystal violet colorimetric assay. BzATP was found to cause a dose-dependent decrease in cell number which was partially reversed by addition of the competitive antagonist KN-62. At a dose of 100  $\mu$ M BzATP, or higher, a significant ( $p < 0.001$ ) reduction in cell number was seen after 48 hours, when compared to control. This decrease in cell number was partially reversed in the presence of 100 nM KN-62 ( $p < 0.001$ , 300  $\mu$ M BzATP compared to 300  $\mu$ M BzATP + 100 nM KN-62). Means  $\pm$  s.e.m. ( $n=9$ ) are shown.

significant. The antagonist KN-62, which is a potent calmodulin kinase inhibitor that has been shown to induce cell death in other cell types, had minimal affect on A375 cell number in isolation.

#### **3.4.6. P2X<sub>7</sub> receptor activation results in apoptosis**

To ascertain whether the anti-proliferative action of P2X<sub>7</sub> receptor agonist BzATP was due to an induction of programmed cell death, the activation of caspase-3 and caspase-7, key enzymes in the process of apoptosis were investigated in A375 melanoma cells. After 6 hours of incubation, BzATP dose-dependently induced an increase in caspase activity. With 300 $\mu$ M BzATP, the caspase activity was increased by 75% when compared to the untreated control (Fig. 3.6).

Figure 3.6



Application of the P2X<sub>7</sub> receptor agonist BzATP causes an increase in caspase 3/7 activity in the A375 melanoma cell line. After incubation with BzATP there was a dose-dependent increase in caspase-3/7 activity. With 300μM BzATP the caspase-3/7 activity was increased by 75% compared to the untreated control ( $p<0.001$ ). Means  $\pm$  s.e.m. (n=9) are shown.

### 3.5. Discussion

Malignant melanoma is the most important cutaneous malignancy since it accounts for over 95% of all deaths from skin disease; the incidence is on the increase and it is resistant to current treatment modalities. ATP is known to inhibit cancer cell growth in a variety of models and through a number of mechanisms (Abraham et al. 2003). One of these is thought to be through the activation of the apoptotic P2X<sub>7</sub> receptor (Chueh, 1993). P2X<sub>7</sub> receptors have been previously described in several cancer types including neuroblastomas (Per et al. 2002), osteosarcomas (Gartland, 2001), squamous cell carcinoma of the skin (Greig, 2003c) and prostate cancer (Calvert, 2004). Recently P2X<sub>7</sub> receptors were described in human melanomas for the first time by immunohistochemical localisation of receptors in specimens of superficial spreading melanoma (Slater, 2003). In this paper we provide evidence for functional P2X<sub>7</sub> receptors in the human melanoma A375 cell line at both the cellular and molecular levels and that activation of this receptor causes apoptosis of melanoma cells in a dose-dependent and reversible manner.

We have shown the synthesis of both P2X<sub>7</sub> receptor mRNA and protein in the human A375 melanoma cell line. To further validate this cell line as a model of purinergic receptor expression in melanoma, we used the same antibody to P2X<sub>7</sub> receptors to confirm the presence of this purinoreceptor subtype on formalin-fixed, paraffin-embedded specimens of melanoma which had been excised from patients. P2X<sub>7</sub> receptors were consistently present in these specimens across differing histogenetic subtypes and this correlates with previous observations.

Currently no selective agonists exist for all the purinoreceptors (Ralevic, 1998) and therefore pharmacological identification relies on the effects of a combination of compounds. ATP itself is active over the entire range of metabotropic

P2Y and ionotropic P2X receptors, BzATP is a potent agonist active at the P2X<sub>7</sub> receptor, although it has also been shown to have effects on other P2 receptors (Khakh et al. 2000; King et al. 2000). KN-62 however, is a potent selective inhibitor of P2X<sub>7</sub>-mediated responses in human cells (Gargett and Wiley 1997). The inhibition of the BzATP induced YO-PRO-1 entry into A375 melanoma cells by KN-62 implies that the effect of BzATP is mediated through P2X<sub>7</sub> receptors.

The effect of incubation of A375 melanoma cells in the presence of BzATP was a statistically significant ( $p < 0.001$ ) decrease in cell number at doses of 100  $\mu$ M or higher. This was dose-dependent and partially reversed by KN-62 and would be expected if the BzATP was acting on an extracellular receptor linked to cell death. Consistent with these findings was the increase in the activity of caspase-3 and caspase-7 enzymes after treatment of cultured melanoma cells with BzATP. Both these enzymes are involved in intracellular pathways mediating apoptosis (Nicholson and Thornberry 1997) and their up-regulation is indicative of an apoptotic mechanism, such as the activation of P2X<sub>7</sub> receptors.

In summary, we have shown for the first time, there are functional P2X<sub>7</sub> receptors in human melanomas and their activation causes a decrease in cell number by apoptosis. Purinergic receptors are distributed widely throughout normal and diseased tissue and it is likely that further subtypes of either P2X or P2Y receptors will be found in malignant melanoma. Targeting P2X<sub>7</sub> receptors is a novel treatment for this disease.

## **Chapter Four**

**Treatment of an *in vivo* model of melanoma with adenosine**

**5'-triphosphate**



#### **4.1. Abstract**

Athymic mice were inoculated with subcutaneous injections of A375 human melanoma cells and were then treated by daily intraperitoneal injections of adenosine 5'-triphosphate (ATP). The volume of tumour, weight of the host animal and final weight of the tumour nodule was measured over the course of the experiment. There was a statistically significant decrease, of nearly 50% by 7 weeks, in tumour volume and weight in treated compared to untreated mice. The weight loss (cachexia) seen in the untreated group was prevented in the treated group. Histological analysis of the excised tumour nodules showed necrosis in the ATP treated tumours, which was not present in the untreated group. The presence of the P2Y<sub>1</sub> and P2X<sub>7</sub> purinergic receptor subtypes, which have previously been proposed as extracellular targets for the treatment of melanoma with ATP, were demonstrated in the excised specimens by immunohistochemistry. This paper provides further support for the use of ATP as a treatment for melanoma.

## **4.2. Introduction**

Malignant melanoma is a very aggressive cancer that originates from melanocytes, the pigment producing cells of the skin. The incidence of malignant melanoma has doubled every 10 years since the 1950's and it is predicted by the year 2010, the lifetime risk of the disease will approach 1 in 50 of the United Kingdom population (Grin-Jorgensen.C.A et al. 1992). Around 1,500 patients in the UK die each year from malignant melanoma. A large proportion of the work load of United Kingdom Plastic Surgeons is the treatment of this disease. This includes primary excision of the cancer, clearance of regional lymph node groups following lymphatic spread of the disease and management of metastatic spread. Unlike other cancers, metastatic (cancer which has spread around the body) melanoma is virtually untreatable by methods of cancer treatment such as surgery, radiotherapy and chemotherapy. Despite significant advances in the treatment of other cancers, the mortality from melanoma has remained unchanged: 66% of patients with metastatic melanoma will die within 5 years. Alternative forms of treatment for this cancer are, therefore, urgently needed.

Interactions between the nervous system and epidermal melanocytes have been suspected on the basis of their common embryological origin from the neural crest but little published work exists to support this. It has been suggested that melanocytes are innervated by autonomic nerves (Hara et al. 1996) with both acetylcholine and noradrenaline acting as transmitter molecules (Hu et al. 2000). Melanomas, which are tumours of melanocyte origin, have also been reported to have an autonomic innervation which is related to their growth and differentiation (Brocker et al. 1991).

In addition to its key role in cellular metabolism, where it acts as a ubiquitous enzyme co-factor and as the key source of the cellular energy unique to phosphate bond formation, the purine nucleotide adenosine 5'-triphosphate (ATP) also functions as a potent extracellular messenger producing its effects via a distinct family of cell surface receptors (Ralevic and Burnstock 1998). ATP was first shown to be a co-transmitter with noradrenaline in sympathetic nerves in smooth muscle cells of the vas deferens (Sneddon and Burnstock 1984). Sympathetic co-transmission has also been clearly demonstrated in a variety of blood vessels (Burnstock 1998). Co-transmission with acetylcholine in parasympathetic nerves has also been proposed in organs such as the urinary bladder (Burnstock et al. 1972; Hoyle and Burnstock 1993).

ATP acts on extracellular receptors which have been cloned and characterised to consist of two families: P2X ion channel receptors with 7 subtypes and P2Y G-protein coupled receptors with 8 subtypes (Abbracchio and Burnstock 1994). ATP acting on these receptors is involved with both rapid signalling in neurotransmission (Dunn et al. 2001) and also long term signalling in cell proliferation (Greig et al. 2003d), differentiation (Ryten et al. 2002) and apoptosis (Humphreys et al. 2000).

It has been shown recently that melanoma cells express P1 purinoceptors (Merighi et al. 2001) and P2X<sub>7</sub> receptors (Slater et al. 2003a). Our work confirms this (White et al. 2005a; White et al. 2005b) and also identifies the presence of other P2 receptors, in particular the P2Y<sub>1</sub> and P2Y<sub>2</sub> subtypes. Our *in vitro* work already performed with purinergic receptor agonists, such as ATP, has shown that they have the ability to reduce melanoma cell number (White et al. 2005a; White et al. 2005b). This is a pilot study to test this further to see if ATP can prevent melanoma cancer growth *in vivo*. Innoculation of MF-1 immunocompromised mice, with A375 melanoma cells is a well established model of cancer research (Allman et al.

2000;Gershwin et al. 1977). Athymic mice are deficient in matured T cell maturity and make excellent models of cancer growth with a reported take of cancer cells of 100% after subcutaneous injection. Therapeutic plasma levels of ATP or other adenosine nucleotides have been shown to be easily achieved by administration through the intraperitoneal route (Rapaport and Fontaine 1989).

The objectives of this pilot study are to establish whether purinergic receptor agonists can prevent or reduce melanoma growth and metastasis in vivo by producing a model of melanoma by subcutaneous injection of A375 melanoma cells into immunocompromised mice. These mice will be treated by intraperitoneal injection of ATP and outcome will be assessed by measurement of tumour volume, tumour weight and animal weight.

### **4.3. Methods**

A375 melanoma cell culture techniques are described in chapters 2 and 3.

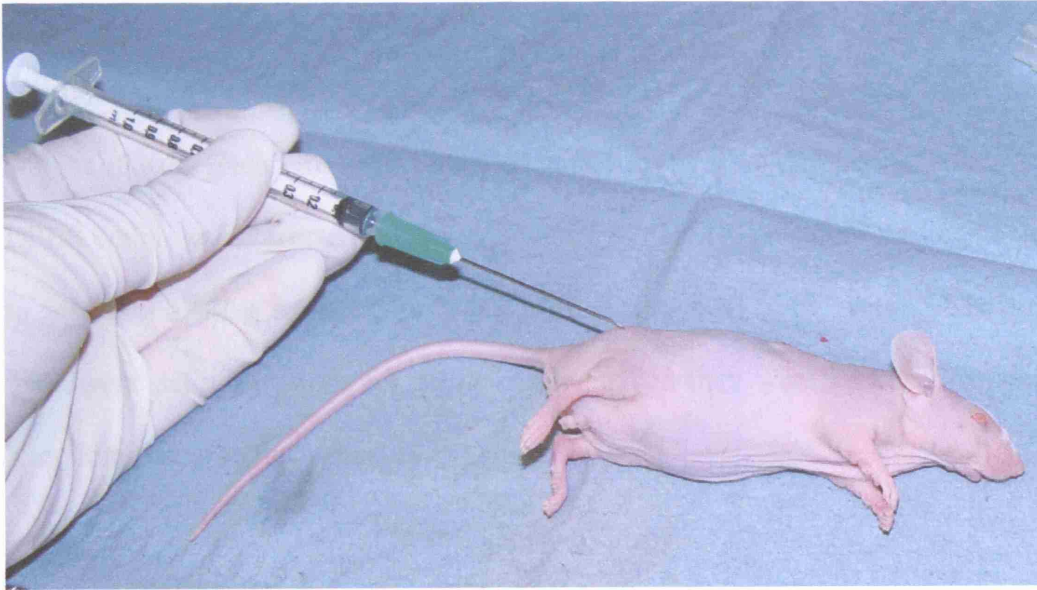
#### **4.3.1. *In vivo* model**

Twenty 6 to 10 week-old male nude mice, strain MF-1, weighing 25-35 g were used in this study. They were kept under barrier conditions in a pathogen-free environment and had excess to food and water *ad liberum*. On day 1 of the experiment  $5 \times 10^6$  A375 melanoma cells were subcutaneously injected, under light general anaesthesia, to induce localized tumour nodules. The injection site was high on the right rear flank; this is illustrated in figure 4.1. The cells were injected in a 100  $\mu$ l suspension consisting of sterile Dulbeccos Phosphate Buffered Saline (DPBS; Sigma, Poole, UK). The animals were then randomly allocated to two groups of 10 which were designated as either the treatment or control group. They were allowed to recover from the procedure. At all times during the full course of the experiment the mice were monitored for adverse effects. These included:

- a) Loss of >15% body weight
- b) Tumour weight > 10% body weight
- c) Ulceration over the tumour
- d) Abnormal behaviour or appearance

This experiment was designed in accordance with the United Kingdom guidelines for the production of solid tumours (UKCCCR 1998).

Figure 4.1 Subcutaneous injection of A375 melanoma cells into an athymic (nude) mouse.



Subcutaneous injection of A375 melanoma cells into an athymic (nude) mouse.

#### **4.3.2. Intraperitoneal injection of ATP**

Ten days post inoculation with A375 melanoma cells the treatment group of 10 mice commenced daily intraperitoneal injections of ATP. The mice had a mean weight of 30 g. Each mouse was injected with 1 ml of 50 mM ATP in a solution of DPBS which was corrected to a pH 6.2 with 1 M NaOH. This dose was equivalent to injecting a 30 g mouse with 1 mg of ATP per g of body weight. The mice underwent daily injections for 39 days after which they were killed by a United Kingdom Home Office schedule 1 method.

#### **4.3.3. Measurement of outcome and statistical analysis**

Three dimensional tumour measurements were performed using callipers and the tumour volume was calculated using the formula:  $\frac{4}{3}\pi[(L/2 \times W/2)^3]$  where L is long axis and W is mean midaxis width (Amizuka et al. 1994). The animals were weighed at regular intervals. After the animals were killed the tumours were removed and weighed. The results were analysed using an analysis of variance (ANOVA) (Excel, Office XP Professional).

#### **4.3.4. Immunohistochemistry**

Immunohistochemistry techniques are described in chapters 2 and 3. In addition double-labeling with P2X<sub>7</sub> receptor antibodies and TdT-mediated dUTP nick end labeling (TUNEL) was performed using a kit (Boehringer Mannheim, Germany). After overnight incubation with P2X<sub>7</sub> receptor antibody diluted to 1:100 as before, sections were washed in PBS and then incubated with the TUNEL reaction mixture for 1 hour at 37°C. As a negative control, sections were incubated with the TUNEL

Label solution only. After further washes in PBS, sections were then incubated with Cy3 conjugated secondary antibody for 1 hour, were washed in PBS and mounted.



#### **4.4. Results**

All animals tolerated the subcutaneous injection of cancer cells and the intraperitoneal injections of ATP; they grew and developed normally in other respects and none of the mice had to be killed before the endpoint of the experiment due to any adverse effect.

The time course of the experiment was 49 days. At day 1 the mice were inoculated with cancer cells, at day 10 the injections of ATP commenced and the mice were killed on day 49. After 21 days there was a clinically obvious development of a palpable tumour nodule in the mice.

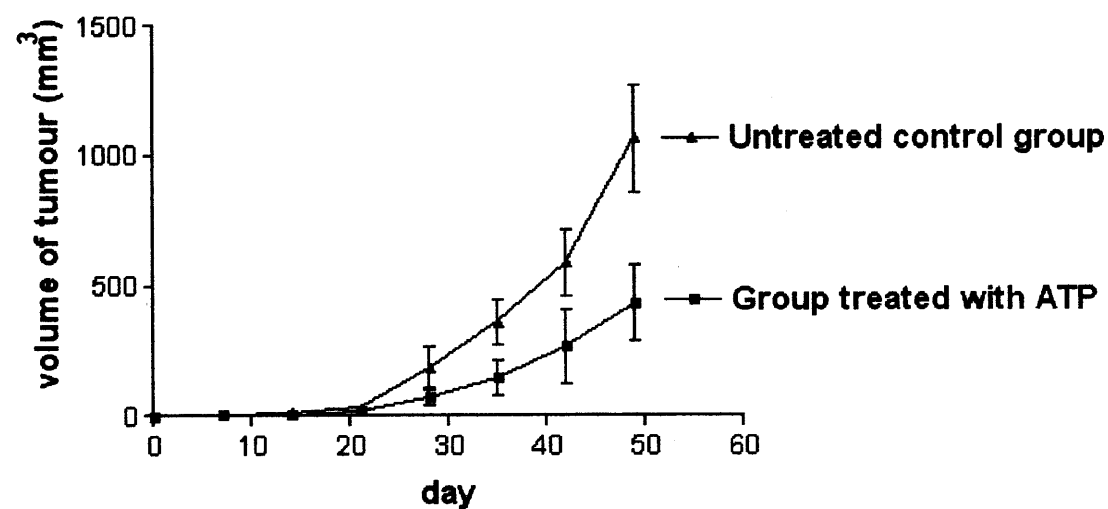
##### **4.4.1. Tumour volume**

There was a statistically significant reduction in tumour volume in the treated group compared to the untreated group ( $p=0.0163$ ), this is illustrated in figure 4.2A. Tumours in both the treated and untreated groups continued to grow during the course of the experiment but the rate of growth of the untreated group was much higher than that of the treated group (fig. 4.2B-C). By day 49 there was nearly a 50% reduction in tumour volume in the treated animals.

##### **4.4.2. Tumour weight**

At the end of the experiment the tumour nodules were excised and weighed (fig. 4.3A). There was a statistically significant reduction in final tumour weight in the treated group compared to the untreated group ( $p=0.0156$ ). The tumours from the untreated group had a mean weight of  $1.92 \pm 0.31$  g compared to the tumours from the treated group which had a mean weight of  $1.15 \pm 0.24$  g (fig. 4.3B).

Figure 4.2 A



There was a statistically significant reduction in tumour volume in the treated group compared to the untreated group ( $p=0.0163$ ). Tumours in both the treated and untreated groups continued to grow during the course of the experiment but the rate of growth of the untreated group was much higher than that of the treated group.

Figure 4.2B-C

**B**

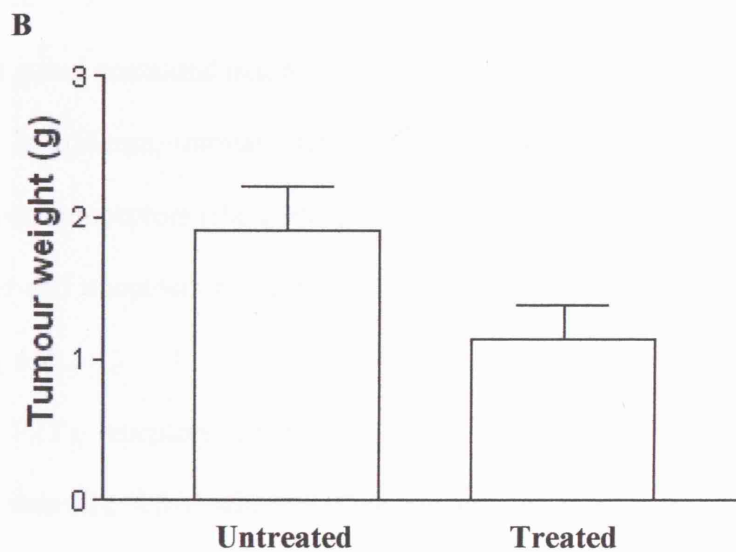
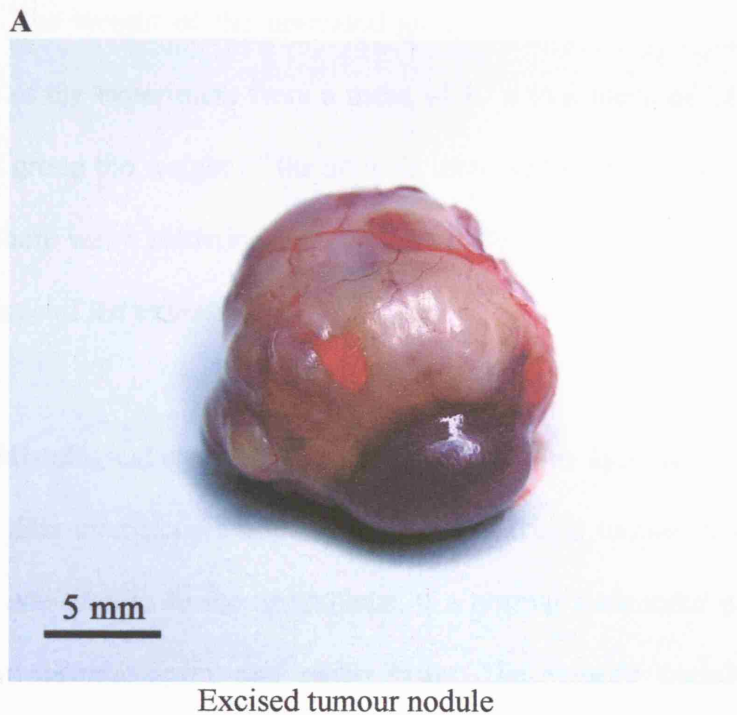


**C**



At 6 weeks post injection there was a visible difference in tumour volume in the treated mice (B) compared to the untreated mice (C).

Figure 4.3



(A) At the end of the experiment the tumour nodules were excised and weighed. (B) There was a statistically significant reduction in final tumour weight in the treated group compared to the untreated group ( $p=0.0156$ ). The tumours from the untreated group had a mean weight of  $1.92 \pm 0.31$  g compared to the tumours from the treated group which had a mean weight of  $1.15 \pm 0.24$  g.

#### **4.4.3. Animal weight**

The weight of the untreated group of animals decreased over the six week course of the experiment from a mean of 30 g to a mean of 28.2 g. However, in the treated group the weight of the animals increased from a mean of 30 g to 31.8 g (fig. 4.4). There was a statistically significant difference in the weight of the animals over the course of the experiment ( $p=0.0038$ ).

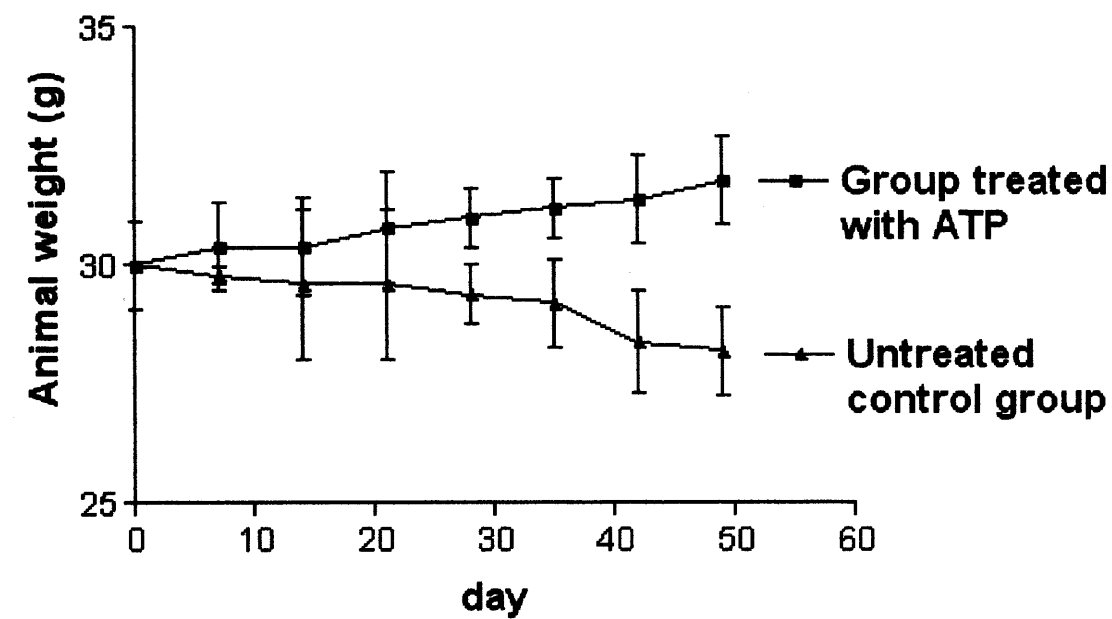
#### **4.4.4. Histological examination of excised tumour specimens**

The microscopic appearance of the excised tumour nodule from the animal model was similar to the appearance of a normal melanoma when examined with a standard haematoxylin and eosin stain. The tumour nodules excised from the untreated group were solid tumours (fig. 4.5A) whereas the specimens from the treated group contained patchy areas of necrosis (fig 4.5B)

In addition, immunohistochemical analysis of the tumour cells for P2X<sub>7</sub> and P2Y<sub>1</sub> purinoreceptors (the 2 receptor subtypes implicated in causing a decrease in cell number and apoptosis respectively) showed the presence of these receptors (fig. 4.6 and fig 4.7).

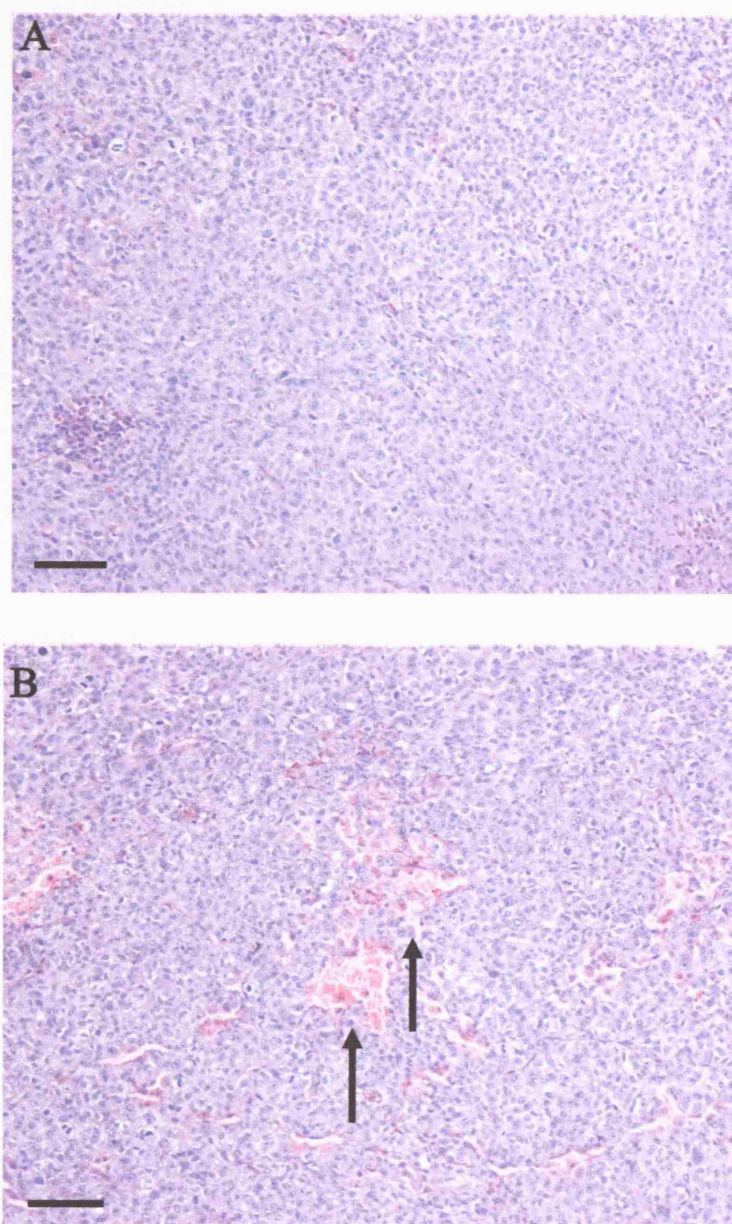
P2Y<sub>1</sub> receptors were only scarcely distributed in the untreated group of melanomas (fig. 4.6A) whereas there was greater P2Y<sub>1</sub> receptor staining in the treated group but this was concentrated around the areas of necrosis (fig. 4.6B). Localisation studies for the P2X<sub>7</sub> receptor and TUNEL (TdT-mediated dUTP nick end labelling) showed extracellular staining of the P2X<sub>7</sub> receptor and cytoplasmic staining for TUNEL (fig. 4.7). TUNEL identifies cells undergoing apoptosis by labelling nuclear DNA fragments that have been cleaved during apoptosis (Gavrieli et al. 1992). There was some overlap of staining, but that TUNEL largely stained the cytoplasm of

Figure 4.4



The weight of the untreated group of animals decreased over the six week course of the experiment from a mean of 30 g to a mean of 28.2 g. However, in the treated group the weight of the animals increased from a mean of 30 g to 31.8 g. There was a statistically significant difference in the weight of the animals over the course of the experiment ( $p=0.0038$ ).

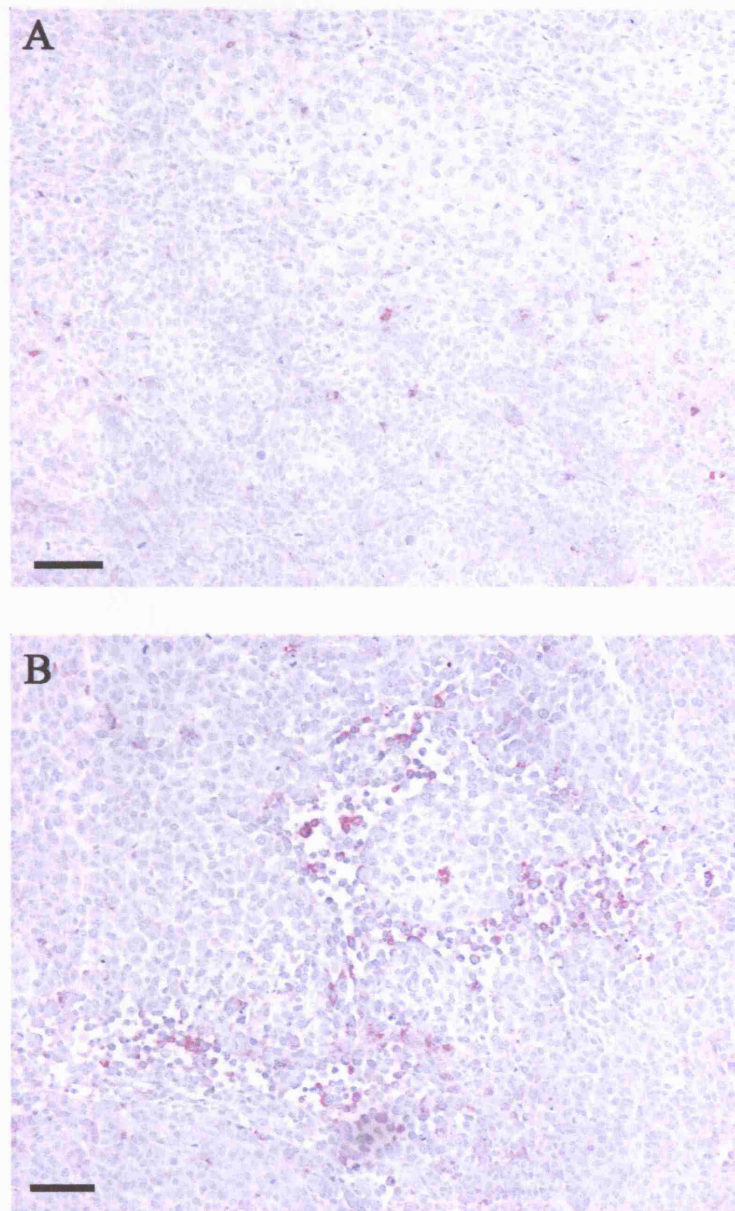
Figure 4.5



Haematoxylin and eosin staining of excised tumour nodules. (A) Solid tumour from an untreated mouse. (B) Tumour from a mouse treated with ATP showing patchy necrosis, illustrated by red staining areas with no nuclear counterstain, examples of which are highlighted with arrows. Bar=250  $\mu$ m.



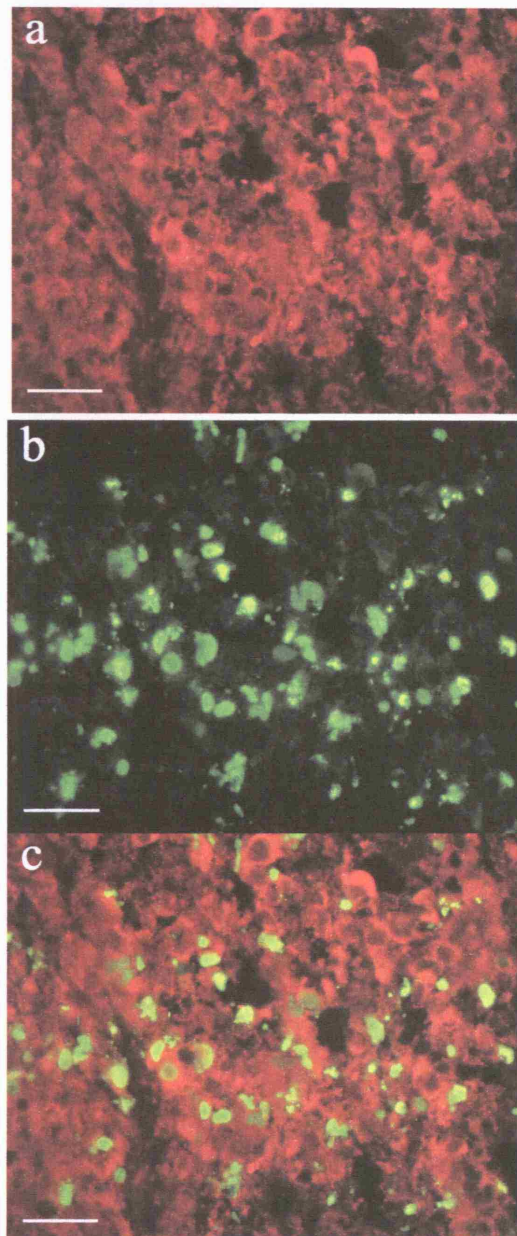
Figure 4.6



Immunohistochemical staining of excised tumour nodules for P2Y<sub>1</sub> receptors. (A) Scant expression of P2Y<sub>1</sub> receptors (pink) in a specimen of untreated melanoma with a haematoxylin nuclear counterstain (purple). (B) Increased P2Y<sub>1</sub> receptor staining in the ATP treated group concentrated around the areas of necrosis. Haematoxylin nuclear counterstain (purple). Bar=250 μm.



Figure 4.7



Localisation of P2X<sub>7</sub> receptors and TUNEL in an excised melanoma from an ATP treated mouse. (a) Red extracellular P2X<sub>7</sub> staining. (b) Green intracellular TUNEL staining. (c) Localisation of extracellular P2X<sub>7</sub> receptors and intracellular TUNEL in ATP treated melanoma tissue undergoing apoptosis. Bar=50  $\mu$ M.

melanoma cells undergoing apoptosis in the specimens from the group which was treated with ATP. P2X<sub>7</sub> extracellular staining was present in both sets of tissue.

#### 4.5. Discussion

We have shown that intraperitoneal injections of ATP inhibits the growth of A375 melanoma cells in an *in vivo* model. The rate of growth of induced tumours was decreased in the group treated with ATP. The final weight of the induced tumour nodule was less in the treated group than the untreated group. Weight loss was prevented in those tumour bearing mice which were treated with ATP.

The intraperitoneal route of administration of ATP is the only practical route of access for giving large doses and volumes of ATP in solution to a small animal. Previous experimental work (Rapaport and Fontaine 1989) has shown that an intraperitoneal injection of 50 mM of ATP gives a circulating plasma level of 5  $\mu$ M five hours post injection, compared to a baseline measurement of 10 nM. The concentrations of ATP needed to produce a decrease in cell number in the previously described *in vitro* studies (White et al. 2005a,b) are higher than the circulating plasma levels in this *in vivo* model but still produce an anti-cancer effect. This can be explained by a single dose of ATP being used in the *in vitro* system compared to multiple daily injections in the *in vivo* model. Also, the efficacy of the anticancer effects of ATP in humans is underestimated in *in vivo* murine studies. This is due to human blood and tissue having a significantly lower level of enzyme activity to breakdown extracellular ATP than a mouse (Rapaport 1988). Intravenous infusions of relatively low levels of ATP into humans has been shown to produce a rapid elevation of circulating ATP levels in both healthy volunteers (Gaba et al. 1986) and cancer patients (Haskell et al. 1996).

A striking finding in our study is the prevention of cancer induced weight loss or cachexia. This has been previously described as an effect of ATP treatment of cancer in both murine models of other cancer types (Rapaport and Fontaine 1989) and clinical trials (Agteresch et al. 2000). In an *in vivo* study such as the work presented

here it is necessary to ask whether the prevention of weight loss is due to the direct effect of ATP acting on cancer cells or is it mediated by another action of ATP in the animal. Possible other mechanisms of action include the up-regulation of the immune system to combat a growing tumour mediated by increased circulating plasma ATP levels. Purines such as ATP have been implicated in priming the immune system and activating immune response cascades (Burnstock 2001). This may be occurring in a whole animal model of cancer leading to a decreased rate of growth of the cancer cells and less weight loss. Also, the liver plays a major role in cancer cachexia. Cancer induced weight loss is mediated by increased catabolism in the liver to provide energy for the rapidly growing tumour cells. This causes increased gluconeogenesis which increases ATP consumption and decreased glycolysis which decreases ATP synthesis. Following intraperitoneal injection, ATP will pass through the liver following its absorption into the enteric circulation. Therefore some of this ATP may be used to reducing weight loss without being associated with its direct effect on the tumour itself.

In this study we have strong histological evidence for ATP acting directly on the tumour. Firstly, the tumours treated with ATP show areas of necrosis which is not present in the untreated groups when examined microscopically after routine H and E staining. Two P2 receptor subtypes have been described to have anti cancer actions in A375 melanoma cells. These are the P2Y<sub>1</sub> and P2X<sub>7</sub> purinoreceptors (see chapters 3 and 4). Very few of the melanoma cells in the untreated group expressed the P2Y<sub>1</sub> receptor, but there were substantially more cells positive for the P2Y<sub>1</sub> receptor in the ATP treated group and these were concentrated around the areas of necrosis. Finally, we have shown that the P2X<sub>7</sub> receptor mediates apoptosis in melanoma cells. In

tumour specimens excised from host mice treated with ATP there was co-localisation of the P2X<sub>7</sub> receptor with TUNEL, a marker of apoptosis.

In summary, ATP has an anticancer activity when used to treat athymic MF-1 mice inoculated with A375 melanoma cells and this action appears to be mediated through P2Y<sub>1</sub> and P2X<sub>7</sub> receptors.

## **Chapter Five**

**Loss of P2X<sub>3</sub> receptor expression is a marker of malignant  
change in melanomas**

### 5.1. Abstract

The extracellular purinergic receptor P2X<sub>3</sub> has been demonstrated on sensory neurons and sympathetic, parasympathetic and enteric ganglia, all tissues derived from the neural crest, and is involved in short-term signalling in neurotransmission. Melanocytes and melanomas, tumours of melanocyte origin, have the same embryological origin from the neural crest. This study investigates the expression of the P2X<sub>3</sub> receptor in cutaneous melanocytic lesions. The expression of the P2X<sub>3</sub> receptor in 10 benign melanocytic naevi and 51 melanomas, of different histological subgroups, was examined using immunohistochemistry. All of the benign naevi stained positive for the P2X<sub>3</sub> receptor, while only 33 of the 51 melanomas were positive. There was no statistically significant correlation between loss of P2X<sub>3</sub> receptor expression and clinical outcome measured by regional lymphatic spread, local recurrence or survival in the melanoma group. However, ulcerated melanomas were significantly less likely to express the P2X<sub>3</sub> receptor than non-ulcerated melanomas ( $p=0.043$ ). This study shows that in malignant melanoma there is loss of P2X<sub>3</sub> receptor expression, particularly in melanomas that have ulcerated. The lack of P2X<sub>3</sub> receptor expression may be useful to distinguish between benign and malignant melanocytic lesions, although it is unlikely to be a useful marker of clinical outcome in established melanomas.

## **5.2. Introduction**

Cutaneous malignant melanoma is an aggressive cancer that originates from melanocytes, the pigment-producing cells of the skin. The incidence of melanoma is increasing (Osborne 2002) and the outcome for patients with advanced disease remains poor (Dreiling et al. 1996). The most important predictive indicator of survival is the Breslow thickness of the primary malignancy (Breslow 1970); with also lymphatic spread and ulceration being indicative of a poor prognosis (Balch et al. 2001a; Balch et al. 2001b). However, some thick melanomas (with a breslow thickness greater than 1 mm) may have a variable prognosis while some thin melanomas (with a breslow thickness less than 1 mm) may be fatal (Soong et al. 1992). Consequently, considerable effort has been directed towards the development of immunohistochemical markers that can be used for prognosis (Li et al. 2002; Mangini et al. 2002), at least 25 such markers have been described which include cell adhesion molecules (Denton et al. 1992), proliferation markers (Smolle et al. 1989), cell cycle regulators (Straume and Akslen 1997) and extracellular transmembrane receptors (Gitay-Goren et al. 1993).

Interactions between the nervous system and epidermal melanocytes have been suspected on the basis of their common embryological origin from the neural crest. It has been suggested that both melanocytes and melanomas are innervated by autonomic nerves with acetylcholine and noradrenaline acting as transmitter molecules (Hara et al. 1996; Hu et al. 2000). The purine nucleotide adenosine 5'-triphosphate (ATP) has a key role in cellular metabolism, where it acts as a ubiquitous enzyme co-factor and as the key source of the cellular energy unique to phosphate bond formation. In addition, ATP is now well established as a potent extracellular messenger, producing its effects via two distinct families of cell surface receptors



designated P2X and P2Y (Ralevic and Burnstock 1998). ATP was first shown to be a co-transmitter with noradrenaline in sympathetic nerves in smooth muscle cells of the vas deferens (Sneddon and Burnstock 1984) and sympathetic co-transmission has also been clearly demonstrated in a variety of blood vessels (Burnstock 1998). ATP acting as a co-transmitter with acetylcholine in parasympathetic nerves has also been proposed in organs such as the urinary bladder (Burnstock et al. 1972; Hoyle and Burnstock 1993).

ATP acts on extracellular receptors which have been cloned and characterised and consist of two families: P2X ion channel receptors with 7 subtypes and P2Y G-protein coupled receptors with 8 subtypes (Abbracchio and Burnstock 1994). ATP acting on these receptors is involved with both rapid signalling in neurotransmission (Dunn et al. 2001) and also long term signalling in cell proliferation (Greig et al. 2003d), differentiation (Ryten et al. 2002) and apoptosis (Humphreys et al. 2000).

The P2X<sub>3</sub> receptor is a ligand-gated ion channel, with intracellular N- and C-termini, two transmembrane spanning domains and a large extracellular loop (Chen et al. 1995). It has a well established role in sensory neurotransmission (Burnstock 2000) and has also been shown to be involved in short-term signalling in sympathetic, parasympathetic and enteric ganglia (Dunn et al. 2001) all tissues which are, along with melanocytes, derived from the neural crest.

Increased expression of other purinergic receptors has recently been suggested as a prognostic marker in cancer. Normal and mildly hyperplastic breast tissue cells were shown not to express the P2X<sub>7</sub> receptor subtype, whereas atypical hyperplastic and cancerous cells were strongly positive for the P2X<sub>7</sub> receptor (Slater et al. 2004). In prostate cancer, expression of the P2X<sub>1</sub> and P2X<sub>2</sub> receptors was claimed to be a marker of early neoplastic change (Slater et al. 2003b). In this study, the expression of

P2X<sub>3</sub> receptors, in formalin-fixed paraffin-embedded benign and malignant melanocytic lesions was investigated using immunohistochemistry.

### **5.3. Materials and methods**

#### **5.3.1. Tissue specimens**

Sixty-one melanocytic lesions from patients with known clinical outcome were studied. Each specimen had been fixed in 10% formal saline and embedded in paraffin. The material included 10 benign melanocytic naevi, 21 superficial spreading melanoma, 18 nodular melanomas, 8 lentigo maligna melanomas, 3 acral lentiginous melanomas and 1 desmoplastic melanoma. For the primary cutaneous melanomas clinical data was collected on Breslow thickness, regional lymphatic spread, recurrence, ulceration and survival at 5 years post-diagnosis.

#### **5.3.2. Primary antibody**

The immunogen used for production of the polyclonal P2X<sub>3</sub> antibody was a synthetic peptide corresponding to 15 receptor-type-specific amino acids (peptide sequence AEKQSTD SGAFSIGH) in the intracellular C-termini of the cloned human P2X<sub>3</sub> receptor, in a similar method to that previously described (Oglesby et al. 1999). The polyclonal antibody was raised by multiple monthly injections of New Zealand rabbits with the peptide.

#### **5.3.3. Immunohistochemical staining**

Paraffin blocks were sectioned at 4 µm on a Reichert-Jung Microtome, and sections were taken on Snowcoat Extra slides (Surgipath, Cambridgeshire, UK), then dried in an oven for 2 hours at 60°C. They were dewaxed and rehydrated using xylene and graded concentrations of ethanol. Antigen retrieval was performed by microwaving for 10 minutes in a solution of 1mM ethylenediamine tetraacetic acid (Tris-EDTA) at pH 9.0. Endogenous alkaline phosphatase was blocked by 20 minute

incubation in 20% acetic acid. Sections were washed and then incubated with avidin D blocking solution, biotin blocking solution and 1:5 normal swine serum (Vector Laboratories).

100 µl of P2X<sub>3</sub> receptor antibody, kept frozen at a stock concentration of 1 mg/ml, was diluted 1:100 and applied for 12 hours at 4°C. 100 µl of biotinylated anti-rabbit antibody (DAKO E0353), diluted 1:200 in DAKO ChemMate diluent was applied for 30 minutes followed by 100 µl of streptavidin alkaline phosphatase (Vector SA5100) diluted 1:200 in DAKO ChemMate for 30 minutes. Vector Red substrate (Vector alkaline phosphatase substrate, SK5100) prepared in 200 mM Tris-HCl (pH 8.2) was then applied for 10 minutes. Positive P2X<sub>3</sub> staining appeared bright pink, nuclei were counterstained purple with hematoxylin. All sections were subsequently dehydrated, cleared and mounted. Negative controls were performed by either omission of the primary antibody or preabsorption of the primary antibody with the corresponding peptide sequence.

#### **5.3.4. Evaluation of results and statistical analysis**

The specimens were divided into two groups: those where P2X<sub>3</sub> receptor staining was present and those where it was absent. Differences in P2X<sub>3</sub> receptor expression in the two groups, for each of the clinical data sets collected, was analysed using the chi-squared test with Yates correction (Excel, Microsoft Office XP Professional).

### **5.3.5. Photography**

The results were analysed using a Zeiss Axioplan high definition light microscope (Oberkochen, Germany) mounted with a Leica DC 200 digital camera (Heerbrugg, Switzerland).

#### 5.4. Results

All 10 (100%) of the benign naevi stained positive for P2X<sub>3</sub> receptors (Fig 5.2) whereas only 33 out of the 51 (65%) melanoma specimens stained positive (Fig 5.3) with 18 of the melanoma specimens with no or minimal staining (Fig 5.4). Internal positive controls were used to ensure that the immunohistochemical staining for each section was successful. This was the positive staining of the keratinocytes in the epidermis which is shown in figures 5.2-5.4 and has been described previously (Denda et al. 2002;Slater et al. 2003).

Each of the histological subtypes of melanoma included specimens that were positive for P2X<sub>3</sub> receptors, the results are summarised in figure 5.1. There were no statistically significant differences between the subgroups, although there was variance in the number of specimens expressing the P2X<sub>3</sub> receptor with comparatively low expression in the superficial spreading (62%) and nodular (61%) group and a high number of specimens positive for P2X<sub>3</sub> in the lentigo maligna group (75%).

The range of Breslow thickness was from 0.1-35 mm with a median value of 1.6 mm. Sixteen out of 25 melanomas (64%) with a Breslow thickness below the median value were positive for P2X<sub>3</sub> receptors while 17 out of 25 melanomas (68%) with a Breslow thickness above the median were positive.

There was a statistically significant correlation between the loss of expression of P2X<sub>3</sub> receptors and ulceration. Ten of the 51 melanoma specimens were ulcerated; 3 of the ulcerated specimens were positive for P2X<sub>3</sub> receptors while 7 were melanomas which did not express P2X<sub>3</sub> receptors. This was compared to 29 of the non-ulcerated melanomas with positive staining and 12 of the non-ulcerated melanomas with negative or minimal staining ( $p=0.043$ ).

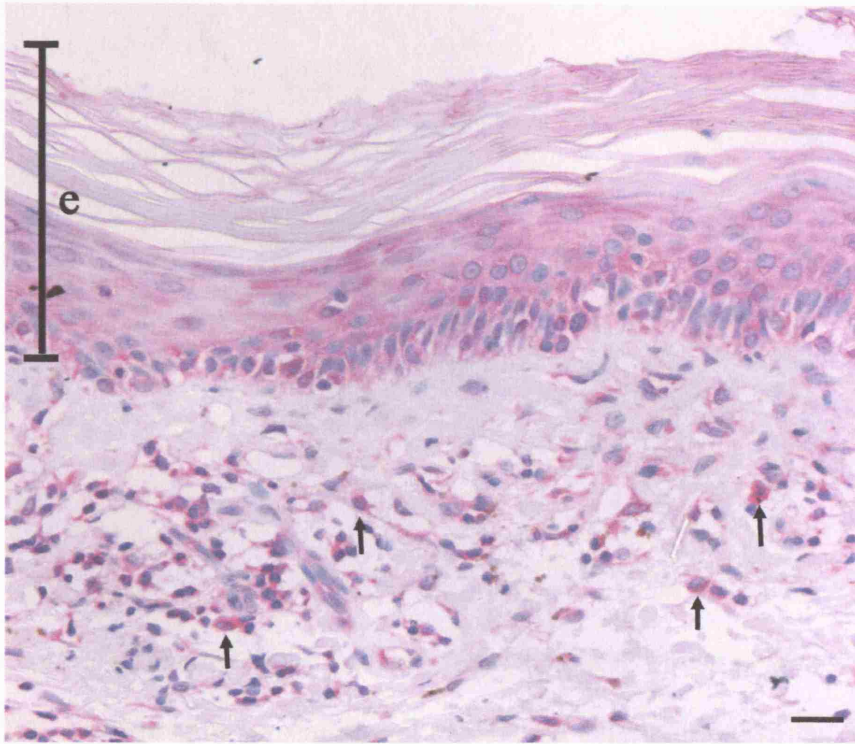
Nine of the 15 melanomas (60%) from patients who were known to have regional lymphatic spread were P2X<sub>3</sub> receptor positive while 24 out of the 36 melanomas (67%) without regional lymphatic spread were positive ( $p=0.89$ ). Those melanomas from patients with known local recurrence stained positive for P2X<sub>3</sub> receptors in 3 out of 7 cases (43%) and 30 out of 44 (68%) specimens with no history of recurrence stained positive ( $p=0.38$ ). Fourteen of the 22 (64%) specimens from patients who died of a cause related to melanoma were positive for P2X<sub>3</sub> receptors while 19 out of 29 (65%) specimens, which were from patients alive at 5 years post-excision, were negative ( $p=0.87$ ).

Figure 5.1

Melanoma	Number of specimens	Number of positive specimens (%)
Superficial spreading	21	13 (62)
Nodular	18	11 (61)
Lentigo maligna melanoma	8	6 (75)
Acral lentiginous	3	2 (67)
Desmoplastic	1	1 (100)
Total	51	33 (65)

Positive P2X<sub>3</sub> immunostaining on paraffin sections of melanoma

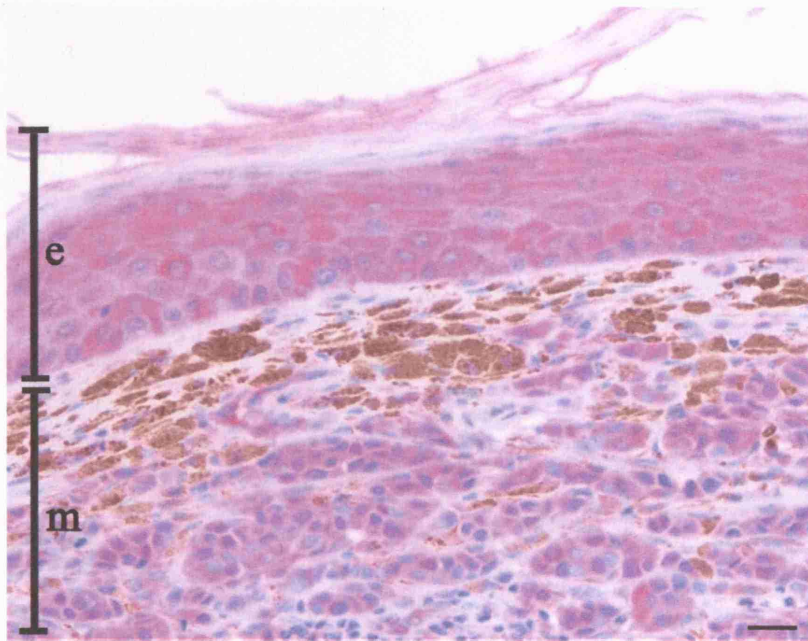
Figure 5.2



An example of positive (pink) P2X<sub>3</sub> receptor immunostaining in a benign melanocytic naevus with purple haematoxylin nuclear counterstain. Examples of positive staining melanocytes are seen. The staining of epidermal keratinocytes for P2X<sub>3</sub> receptors is an internal positive control. E=epidermis. Bar= 250  $\mu$ M.

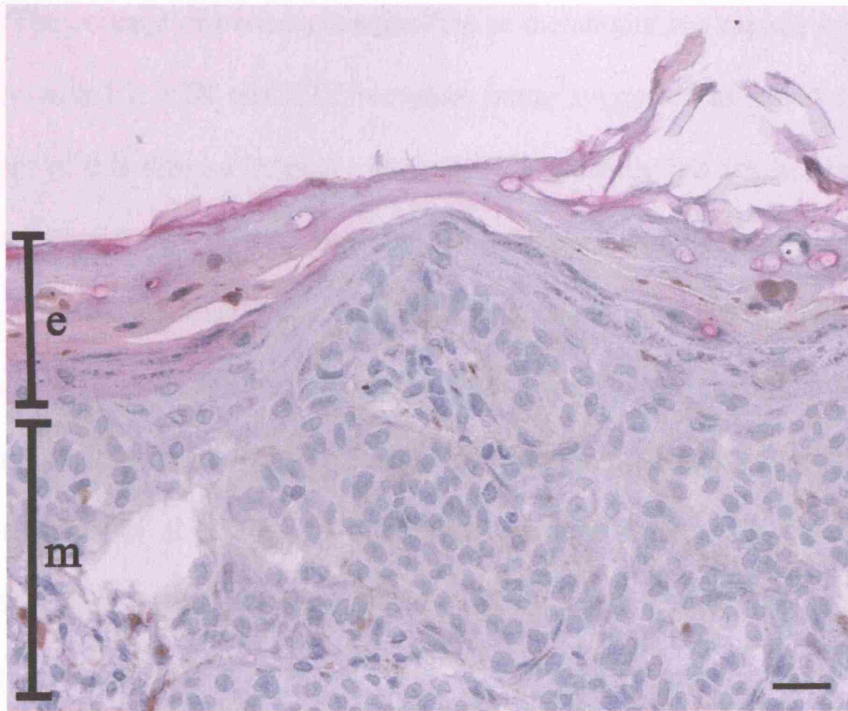


Figure 5.3



An example of predominantly positive immunostaining in a specimen of melanoma. Pink P2X<sub>3</sub> receptor immunostaining of melanoma cells and epidermal keratinocytes with purple haematoxylin nuclear counterstain is shown. E=epidermis. M=melanoma. Bar= 250  $\mu$ M.

Figure 5.4



An example of negative immunostaining in an ulcerated specimen of melanoma. Pink P2X<sub>3</sub> receptor immunostaining of epidermal keratinocytes and negative staining melanoma with purple haematoxylin nuclear counterstaining. E=epidermis. M=melanoma. Bar= 250  $\mu$ M.

## 5.5. Discussion

The concept of purinergic signalling in melanoma is a rapidly expanding field of study with P1, P2X and P2Y receptors being suggested as novel targets for the treatment of this disease (Merighi et al. 2002). No study has yet been undertaken to assess whether there may be a diagnostic role for purinergic receptor expression.

The extracellular P2X<sub>3</sub> purinergic receptor is expressed ubiquitously in all tissues derived from the neural crest, where it plays an important role in initiating signalling in sensory nerves (Burnstock 2000) and in neurotransmission in autonomic ganglia (Dunn et al. 2001). In this study we have shown, for the first time, the expression of this receptor in melanocytes and melanomas using immunohistochemistry.

A previous report was unable to show the presence of P2X<sub>3</sub> receptor in a series of formalin-fixed, paraffin embedded melanomas (Slater et al. 2003a). Several factors influence immunohistochemical study results and these include the source of the antibody, antigen retrieval methods and concentrations used (Mangini et al. 2002). In this study a different antibody and method was used. An internal positive control, which was staining of the keratinocytes of the epidermis (Denda et al. 2002; Slater et al. 2003a), confirmed the efficacy of the technique used in our study.

Positive staining was present in all 10 naevi specimens examined. Fifty-one specimens of melanoma were also assessed but only 33 of these had positive staining. This suggests a loss of expression of the extracellular receptor as melanocytes undergo malignant change, and implicates the P2X<sub>3</sub> receptor as having a role in cell signalling and the regulation of melanocytes which is down regulated or lost in melanomas. Further studies will be needed to assess whether the P2X<sub>3</sub> receptor plays a functional role in the normal homeostatic mechanisms of melanocytes.

The P2X<sub>3</sub> receptor was first described in sensory neurons (Chen et al. 1995) and was then shown to be present in autonomic ganglia (Dunn et al. 2001). Due to their common embryological origin, the presence of P2X<sub>3</sub> receptors in melanocytes and melanomas is understandable. The presence of the P2X<sub>3</sub> receptor in other tissue types has also been described. There is evidence of P2X<sub>3</sub> receptor labelling of endothelial cells of vessels (Burnstock 2002b) as well as the epithelial cells of the thyroid (Glass and Burnstock 2001). In addition, this is the third report to date that shows P2X<sub>3</sub> receptors are present in keratinocytes (Denda et al. 2002; Slater et al. 2003a). This is a surprising finding considering the known expression and function of this subtype of purinergic receptor, especially when other P2 receptor subtypes have been described in keratinocytes with designated roles (Greig et al. 2003d). Whether the P2X<sub>3</sub> receptor has a function in the homeostasis of normal epidermis is unclear.

Each of the five histological subtypes of melanoma studied included specimens that were positive for P2X<sub>3</sub> receptors. Even though there was no statistically significant difference, there was a decrease in expression of the P2X<sub>3</sub> receptor in superficial spreading and nodular subgroups and increased expression in the lentigo maligna subtype group. This difference may be due to the development of lentigo maligna melanoma from pre-existing melanocytic lesions. With the small number of specimens in the rarer acral lentiginous and desmoplastic subgroups it is not possible to draw any conclusions other than these histological subtypes are capable of P2X<sub>3</sub> receptor expression.

Ulcerated melanomas were found to be less likely to express the extracellular P2X<sub>3</sub> receptor than non-ulcerated melanomas. Melanomas which ulcerate carry a worse prognosis and this finding suggests the loss of the P2X<sub>3</sub> maybe a feature of aggressive disease or disease progression, which is supported by the loss of P2X<sub>3</sub>

receptors by melanomas compared to benign melanocytic naevi. Other clinical outcome data from each of the melanoma specimens, which were collected and compared with P2X<sub>3</sub> receptor expression, were regional lymphatic spread, local recurrence, and survival. However no statistically significant result correlating loss of P2X<sub>3</sub> receptor expression and clinical outcome was found.

In this study we have shown for the first time the expression of the P2X<sub>3</sub> receptor in both benign naevi and melanomas. We have also demonstrated the loss of P2X<sub>3</sub> receptor expression in some melanomas. This is the first example of a cancer type losing the expression of a purinergic receptor as normal tissue undergoes malignant change. Ulcerated melanomas are more likely not to express P2X<sub>3</sub> receptors than non-ulcerated melanomas. However P2X<sub>3</sub> receptor expression has not been shown to be an indicator of clinical outcome.

## **Chapter Six**

### **Discussion**

Malignant melanoma is an important type of cancer. This is because its incidence is on the increase and there are no successful treatments for the disease once it has metastasised from the primary site. Due to this malignant melanoma is the subject of intensive study to understand this disease process and to develop potential new treatments.

A variety of different approaches have been used in this thesis to study purinergic signalling in melanoma. In this general discussion I summarise the major findings and discuss some advantages and disadvantages of the experimental methods used and how these approaches have been used to build up a picture of the role of purinergic signalling in melanoma. Then, whether the presence of a purinergic messenger system is of functional significance is explored. This is followed by a discussion of the role of the trophic effects of purinergic signalling in normal physiological and diseased states. Finally there is a discussion of the therapeutic potential of purinergic signalling in cancer and future directions to develop the work presented in this thesis into clinical practice are explored.

### **6.1. Major findings of this thesis**

Both ionotropic P2X and metabotropic P2Y purinoreceptors have been extensively studied in this thesis. In chapter 2 which focuses on the P2Y purinoreceptors; the functional expression of P2Y receptors is described. P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub> are characterised in melanoma tissue and cells and P2Y receptors are shown to regulate melanoma cell proliferation with activation of the P2Y<sub>1</sub> receptor subtype causing a decrease in cell number and activation of the P2Y<sub>2</sub> receptor subtype causing an increase in cell number.

Chapter 3 explores the role of ionotropic P2X receptors in the regulation of melanoma cells. The presence of functional P2X<sub>7</sub> receptors is demonstrated in melanomas. Activation of this receptor is shown to cause an activation of the caspase enzyme cascade leading to cell apoptosis and an overall decrease in cell number.

In chapter 4, purinergic signalling as a potential therapeutic target for the treatment of melanoma *in vivo* is investigated. Nude (athymic) mice inoculated with A375 human melanoma cells are used as the experimental model of malignant melanoma. Systemic treatment of this model with ATP caused a reduction in the rate of growth of melanoma and this is likely to be mediated by P2Y<sub>1</sub> and P2X<sub>7</sub> receptors, the presence of which are demonstrated histologically in the treated melanoma specimens.

The value of purinoreceptors as diagnostic markers is examined in chapter 5. Using immunohistochemistry, a large number of both benign and malignant pigmented lesions of different histological subtype are examined. There is a significant loss of expression of the P2X<sub>3</sub> receptor in melanomas when compared to benign pigmented naevi. In addition ulcerated malignant melanomas, which have a worse prognosis, are less likely to express P2X<sub>3</sub> receptors than other melanomas.

## **6.2. Are the techniques used valid and useful tools for the study of purinergic signalling in melanoma**

The experimental data presented in this thesis is drawn from studies on the A375 melanoma cell line and paraffin embedded specimens of excised human melanomas. These have been studied using immunohistochemistry, reverse transcriptase polymerase chain reaction, cell number assays, intracellular calcium level assays, caspase activity assays and an *in vivo* model. This combination of



techniques has enable the study of purinergic signalling in melanoma at a molecular (RT-PCR), cellular (cell count, FLIPR, caspase activity), tissue (immunohistochemistry) and whole animal level.

At a molecular level, reverse transcriptase polymerase chain reaction is a technique where RNA being expressed by a cell is isolated, transcribed back into DNA which is then amplified to give a much larger volume of DNA using a primer (small sequence of DNA) which is specific for each purinergic receptor subtype. This DNA product is then identified by running on an agarose gel to give an expected band length. This technique has enabled us to show melanoma cells express RNA for purinoreceptors. A further technique to confirm the accuracy of RT-PCR, which was not used in this project, is sequencing of the DNA product (Schwiebert et al. 2002). This involves identifying the nucleic acid sequence of the DNA product and comparing it to the expected DNA product for the primer used. Due to the high sensitivity of RT-PCR it can normally only be used on mRNA extracted from non-contaminated cells, ie a single cell line, as mRNA extracted from tissues consisting of different cell types could easily become contaminated. One method which may be able to solve this is laser micro dissection (Going and Gusterson 1999). This is a technique where a single cell is dissected from surrounding tissues under a microscope using a laser. This cell can then be investigated individually. Using this technique we may have been able to perform RT-PCR analysis on the excised specimens of human melanomas as well as the melanoma cell line.

The RNA is then transcribed by cells into protein. This protein expression is studied using immunohistochemistry. In this technique antibodies which are specific to the three dimensional structure of the purinergic receptor protein are used which bind to the receptors and are then localised using fluorescent or enzyme based

imaging. This technique can be used with either tissue specimens (immunohistochemistry) or cells grown in culture (immunocytochemistry). The antibodies used in this study were either commercially available P2Y antibodies produced by Alomone Laboratories (Tel Aviv, Israel) or P2X antibodies which were a gift from Roche Bioscience (Palo Alto, USA). Both these groups of antibodies have been used previously to describe P2 receptor expression in human tissues (Greig et al. 2003d). To study protein expression in addition to immunohistochemistry a western blot could have been used (Wang et al. 2004). This is a technique in which protein is extracted and purified from cells. It is then run along a cellulose sheet to separate proteins of different length. The antibody specific for the purinergic receptor is then applied to the agarose sheet and visualised at the expected size. The advantage this has over immunohistochemistry is there is less background staining and non specific binding of the antibody so there is less chance of a false positive. However, it can only be used in cultured cell lines and not tissue samples to prevent contamination with over cell types and does not provide topographical data such as the position of the staining in cells (i.e. on the cell membrane).

Once the presence of purinergic receptors has been established the next step is to establish whether they are functional; i.e. whether they cause a response within the cell to stimulation by ATP or a similar compound. This was assessed using the Fluorometric Imaging Plate Reader. Changes in intracellular calcium levels allowed us to demonstrate functional P2Y receptors and YO-PRO-1 uptake allowed us to demonstrate functional P2X<sub>7</sub> receptors. These responses were shown to be mediated by both ATP and receptor subtype specific agonists and were partially blocked by specific antagonists. The cell signalling pathways initiated by the activation of purinergic receptors were also investigated further. P2Y receptors were shown to be

G-proteins coupled to Phospholipase C by using a PLC inhibitor and the apoptotic P2X<sub>7</sub> was shown to be linked to an apoptotic caspase enzyme cascade using a caspase 3/7 assay. The trophic effect of activation of the functional P2 receptors we described was studied in both an *in vitro* and *in vivo* model which assessed the change in cell number. In the *in vitro* model the change in cell number was recorded using a colourimetric assay which enabled the counting of large numbers of cells rapidly. The *in vivo* model enabled us to measure tissue growth rather than cell growth. This was done by measuring both tissue volume and weight and also allowed us to collect whole animal data about the systemic effect of treating melanomas with ATP.

Other techniques could have been used to make some of the experiments described in this thesis even more robust. In addition to measuring change in cell number there are several well established techniques used to measure cell growth *in vitro* in response to treatment with biologically active compounds. The two most commonly used in cancer research are the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and radiolabelled [3H] thymidine uptake. The MTT assay (Mosmann 1983) is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilizing the cells by the addition of a detergent results in the liberation of the crystals which. The number of surviving cells is directly proportional to the level of the formazan product created. The colour can then be quantified using a simple colourimetric assay.

The [3H] thymidine incorporation proliferation assay involves the uptake of radiolabelled thymidine into living cells, which is then incorporated into the cell itself. The cells are then treated and the amount of radioactivity in the treated and untreated

controls cells are then measured in a scintillation counter to give an estimate of the cell number (Morrone et al. 2003).

In addition, flow cytometry could also have been employed. Flow cytometry employs instrumentation that scans single cells flowing past excitation sources in a liquid medium (Zhang et al. 2004). The technology can provide rapid, quantitative, multiparameter analyses on single living (or dead) cells based on the measurement of visible and fluorescent light emission. Flow cytometry is a widely used method for characterizing and separating individual cells by analysis of which part of the cell cycle they are in.

All these techniques have been validated and have been previously used in peer reviewed publications studying purinergic signalling in cancer. RT-PCR analysis for P2 receptors has been used in endometrial cancer cell lines (Katzur et al. 1999) and oesophageal squamous cell carcinoma biopsies (Maaser et al. 2002). Immunocytochemistry has demonstrated the presence of purinergic receptors in prostate cancer cell lines (Calvert et al. 2004) and immunohistochemistry has elucidated purinergic receptors in excised specimens of human basal and squamous cell carcinomas of the skin (Greig et al. 2003c). Western blots have been used to establish the presence of P1 receptors in A375 melanoma cells (Merighi et al. 2002). The measurement of changes in cell number after treatment with purinergic agents has been described in ovarian cancer cells using [<sup>3</sup>H] Thymidine incorporation (Schultze-Mosgau et al. 2000), using a crystal violet assay in colorectal carcinoma cells (Hopfner et al. 1998) and using MTT in melanoma cells (Merighi et al. 2002). Changes in intracellular calcium levels in endometrial and oesophageal cancer cell lines have been demonstrated in response to activation of purinergic receptors (Katzur et al. 1999; Maaser et al. 2002). Flow cytometry has been used to study P1 receptors in

melanoma cell lines (Merighi et al. 2002). This is however the first report of using YO-PRO-1 to show functioning P2X<sub>7</sub> receptors in any human cancer type.

### **6.3. Is purinoreceptor expression in melanoma of functional significance?**

Studying melanoma cells *in vitro* enabled us to show that extracellular nucleotides can regulate changes in cell number through activation of purinergic receptors. The pharmacology of the responses seen and the expression of mRNA and protein for P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2X<sub>7</sub> receptor subtypes strongly suggested that multiple P2 receptors were involved and that they modulated cell function and number through different mechanisms.

There is considerable evidence to suggest that melanoma tissue is heterogenous and it is not clear whether all cell populations express the same subpopulations of purinergic receptors. The immunohistochemical analysis of the A375 melanoma cells showed a homogenous expression of receptor subtypes but this was not present in the immunohistological staining of excised melanoma specimens. P2Y<sub>2</sub> receptors were just seen on the proliferating margins of the melanomas (chapter 2), P2X<sub>7</sub> receptors were seen in greater than 75 % of cells but its presence was not universal (chapter 3) and the P2X<sub>3</sub> receptors were seen in decreasing numbers in melanomas of increasingly poor prognosis (chapter 5). As all cancer tissue is anaplastic by definition these findings are not unexpected but do raise the question of whether melanomas use purinergic signalling as part of their pathophysiological homeostatic mechanisms and whether these purinergic receptors are a genuine target for cancer therapy.

The significance of functional purinergic receptors in melanomas is supported by the consistency of receptor subtype expression at molecular, cellular and tissue

levels and further supported by the *in vivo* experiments described in chapter five. The systemic effects of high dose ATP on melanomas are those expected based on the *in vitro* studies and the presence of the two purinergic receptor subtypes implicated in reducing cell growth and apoptosis (P2Y<sub>1</sub> and P2X<sub>7</sub>) are clearly demonstrated using immunohistochemistry.

The basis of this thesis is that ATP has a direct anticancer activity due to its action on cell membrane P2 receptors. This is clearly shown in *in vitro* experiments but once systemic ATP is administered into an animal model other possible anticancer effects have to be discussed. Cancer cachexia in humans and experimental animals is due to alterations in energy metabolism. Gluconeogenesis from lactate, three carbon amino acids and glycerol which are the respective products of tumour, muscle and adipose tissue catabolism have been considered as the source of the enormous energy expenditure that occur in the liver during cancer cachexia (Stein 1978). Gluconeogenesis is costly in that 6 ATP molecules are required to produce one molecule of glucose. Glycolysis of these glucose molecules in growing cancer cells yields only 2 molecules of ATP per molecule of glucose. This leads to depletion of ATP pools in the liver, circulating red blood cells and plasma and this in turn leads to the progressive weight loss seen in advanced cancer patients. These alterations in glucose metabolism have been demonstrated with radiolabeled precursors in human cancer patients suffering from progressive weight loss (Holroyde and Reichard, Jr. 1986). Furthermore, the significant depletion of host visceral energy stores by a growing tumour has been demonstrated in experimental animals (Inculet et al. 1987). Along with the decline in hepatic ATP pools in cachexia tumour animal models, severe declines in skeletal muscle ATP pools were demonstrated. Despite this evidence on the systemic effect of ATP on weight loss the majority of the anticancer

effect of ATP is likely to be through the direct action of ATP on cell membrane P2 receptors mediating trophic effects.

As well as a trophic role for P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2X<sub>7</sub> receptors further evidence for purinergic receptors regulating the growth of melanomas is provided in chapter 6 concerning the P2X<sub>3</sub> receptor. Positive staining was present in all 10 naevi specimens examined. Fifty-one specimens of melanoma were also assessed but only 33 of these had positive staining. This suggests a loss of expression of the extracellular receptor as melanocytes undergo malignant change, and implicates the P2X<sub>3</sub> receptor as having a role in cell signalling and the regulation of melanocytes which is down regulated or lost in melanomas. Further studies will be needed to assess whether the P2X<sub>3</sub> receptor plays a functional role in the normal homeostatic mechanisms of melanocytes.

#### **6.4. Long term trophic actions of purinergic signalling in cancer and other tissue types**

The initial research focus of purinergic signalling was the role of extracellular nucleotides and nucleosides in neurotransmission; in particular the role of ATP being released by autonomic neurones regulating secretion, smooth muscle contraction and the regulation of vascular tone (Burnstock 1997). However, there is now growing recognition that purinergic receptors have major long term trophic actions in development, regeneration and pathophysiological processes (Abbracchio and Burnstock 1998; Burnstock 2002a).

The trophic effect of innervation of an organ by neurons is most evident in skeletal muscle where division of a motor nerve proximal to the anatomical point of innervation leads to atrophy of that muscle. It is increasingly becoming evident that

extracellular nucleotides and nucleosides also act as trophic factors in both the central and peripheral nervous system (Neary et al. 1996; Neary 2000). P2 receptor subtypes are expressed on neurons, glial and endothelial cells where they mediate strikingly different effects. These range from induction of cell differentiation and apoptosis, mitogenesis and morphogenesis, to stimulation of synthesis and release of cytokines and neurotrophic factors under both physiological and pathological conditions.

Purinergic receptors have trophic roles in the migration, proliferation and cell death of vascular smooth muscle and endothelial cells (Burnstock 2002). There is a synergistic action of purines and pyrimidines, released from endothelial cells, platelets and sympathetic nerves, with growth factors promoting cell proliferation. ATP is also released from damaged cells in atherosclerosis, hypertension, restenosis, and ischaemia; this is coupled to evidence that vascular smooth muscle and endothelial cells proliferate in these pathological conditions.

Evidence has been presented that purinergic receptors have a trophic role in skeletal muscle development and differentiation (Ryten et al. 2002). P2X<sub>5</sub> receptors are present on skeletal muscle satellite cells and ATP activation of a P2X receptor inhibited proliferation and stimulated markers of cell differentiation.

Most recently the trophic effect of ATP on epidermal keratinocytes has been described (Greig et al. 2003a; Greig et al. 2003b; Greig et al. 2003d). In this work P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2X<sub>5</sub> and P2X<sub>7</sub> receptors are shown to be present and to mediate different trophic effects. P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors were present in different layers of the epidermis and appeared to regulate cell proliferation. P2Y<sub>1</sub> receptors were also present in fetal epidermis but were absent in basal and squamous cell cancer lines (tumours of keratinocyte origin). This suggests that the trophic roles of P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors with respect to proliferation are complex and also vary with cell type.



The trophic role of the P2X<sub>5</sub> receptor in epidermal homeostasis was found to be the regulation of cell differentiation. There was colocalisation of P2X<sub>5</sub> receptors and markers of cell differentiation; ATP $\gamma$ S, a potent agonist of P2X<sub>5</sub> receptors, caused a decrease in keratinocyte number which was postulated to be due to changing the keratinocyte cell cycle to a state where they were differentiating so they could no longer proliferate.

Finally, P2X<sub>7</sub> receptors were shown to mediate terminal differentiation, a specialised form of cell death found in the epidermis. P2X<sub>7</sub> receptors were colocalised with markers of apoptosis and BzATP, a specific P2X<sub>7</sub> receptor agonist, caused a substantial decrease in cell number.

With an increasing body of evidence suggesting a role for purinergic signalling in the trophic regulation of both normal and pathological tissues the regulation of cellular proliferation, differentiation and death in cancer becomes an important field of study.

#### **6.5. Therapeutic potential of purinergic signalling in cancer and future directions of study**

The end point for any research aimed at human cancer therapy is the introduction of a new treatment into clinical practice. For this to be done the new treatment must be effective, have few side effects and have a known mechanism of action. Normally this sequence of events would begin with the mechanism of action being described in an *in vitro* study, then the effectiveness would be recorded in an *in vivo* study and then efficacy and side effects would be studied in a phase I and II clinical trials. However, with respect to purinergic signalling and cancer these events have happened in a different sequence.

The anticancer activity of adenine nucleotides was first described in 1983 (Rapaport 1983). These *in vivo* studies of the systemic use of ATP to treat mice inoculated with cancer cells clearly showed that daily intraperitoneal injections of ATP significantly inhibited tumour growth, prolonged survival time and inhibition of weight loss (cachexia). In addition a synergistic action when administered with established chemotherapeutic agents has been demonstrated.

This work was then followed by preliminary clinical trials studying whether ATP can be used as a therapeutic agent in the treatment of human patients with cancer. Many new cancer therapies become available to patients through clinical trials. Treatment studies that involve drugs or invasive procedures are categorized by phase. Phase I studies generally establish whether a treatment is safe and at what dosages. Phase II studies assess the efficacy of treatments after their safety and feasibility has been established in Phase I studies. Phase III studies compare effective treatments from phase II studies to currently accepted treatments. Phase IV studies, which collect and compare data on established treatments, are used to continually monitor outcome once a new treatment is established. Studies can also be categorized as adjuvant and neoadjuvant. Adjuvant studies evaluate treatments that try to prevent the recurrence of cancer after a patient has become clinically free of disease (for example following the surgical excision of a melanoma and surgical lymphadenectomy following metastatic spread to a regional lymph node basin). Neoadjuvant studies evaluate treatments designed to reduce the extent of tumour to a point where it can be definitively treated by therapies that are considered standard (for example surgical excision).

Phase I studies are the initial clinical tests of new treatments. The major purpose of a Phase I study is either to define a safe dose and schedule of agent or

combination of agents or to evaluate the feasibility of combining treatment modalities. Phase I studies are usually designed based on promising preclinical data, such as *in vitro* cytotoxicity in cancer cell lines and safe administration with reproducible anticancer effect in animals, provided a stable and safe formulation of the agent is available. Phase II studies assess the efficacy of a new cancer agent, a new combination of agents, or a new modality of therapy. Using the dosage (dose and schedule) found to be safe in phase I studies, the new treatment is given to groups of patients with one type of cancer or related cancers. Further determination of toxicity is the second major purpose of phase II studies. In most cases, fewer than 100 patients have ever received the new treatment prior to phase II testing. Phase II studies normally involve several trials that collectively treat hundreds of patients with many types of cancer. Unusual or chronic toxicities, which are often missed entirely in phase I testing, may appear during phase II testing; this is considered when deciding whether the agent should be further evaluated in phase III studies. Phase III studies are designed to compare one or more treatments. A new drug or drug combination may be tested against one of proven efficacy. Phase III studies often have multiple endpoints. Overall and disease free survival are nearly always endpoints; differences in response rates, toxicity, patterns of recurrence, and quality of life might also be endpoints. At the conclusion of a properly designed and conducted phase III study, the new drug or drug combination will be found to be inferior, equivalent, or superior to the standard treatment in respect to the major endpoints. The degree of difference will be known and statistical significance will be estimated.

Three ATP cancer trials have been published. The first was a phase I/II trial of intravenous ATP for 14 patients with advanced, inoperable non-small cell lung cancer (Haskell et al. 1996). This trial established that prolonged infusions of ATP are

feasible with acceptable toxicity and that 50 µg/Kg per minute is both the maximally tolerated dose and the most appropriate dose rate for subsequent phase II testing of 96 hour infusions of ATP in patients with advanced cancer.

The second human clinical trial was a phase II multicentre study of 15 patients with non-small cell lung cancer patients (Haskell et al. 1998). Although no significant cancer shrinkage was seen the majority of patients exhibited stable disease after treatment with ATP and there were beneficial effects in terms of weight gain.

The third trial (Agteresch et al. 2000; Agteresch et al. 2003) was a randomized phase III trial of 52 patients with non-small cell lung cancer who had not responded to previous chemotherapy or radiotherapy. They were divided into 2 groups a control and an ATP treated group. This study showed that ATP as a single therapy did not lead to cancer regression but it may prolong survival and prevent weight loss in patients. The published trials to date have established systemic administration of ATP as a safe agent, which may increase median survival and that it has a future place as a useful anticancer agent.

The *in vitro* analysis of the action of ATP on cancer cells followed rather than preceded the clinical trials. All the previous clinical trials used the generic P2 agonist ATP. With the more recent establishment of P2 receptor subtypes and a clearer idea of their individual functions a new avenue of investigation has opened up. This is targeting of specific P2 receptor subtypes with specific agents rather than the use of ATP. An example of this would be targeting of P2Y<sub>1</sub> and P2X<sub>7</sub> receptors in melanomas. Further examples of specific P2 receptors influencing other cancer types are given in the introduction. A potential criticism of the use of ATP to treat cancer is that due to the widespread distribution of purinergic receptors it will have multiple side effects as purinergic receptors outside the target cancer will be affected.

Combining specific agonists for P2 receptors expressed by the target cancer cells with the knowledge from the clinical ATP trials that minimal side effects can be obtained with a therapeutic dose of ATP starts to negate this argument.

#### **6.6. What is the way forward for the therapeutic manipulation of purinergic signalling in melanoma?**

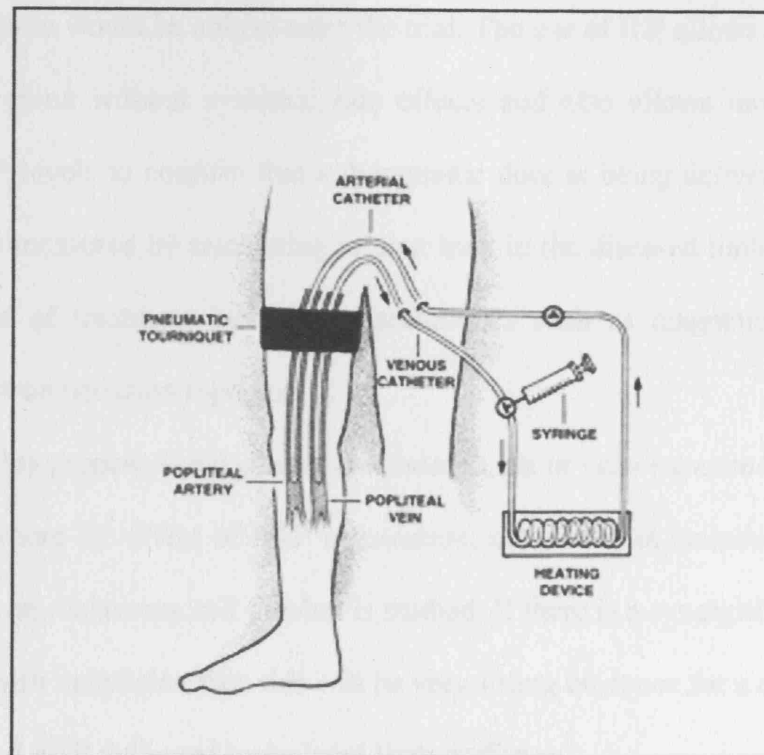
The previously published trials on the use of ATP as a therapeutic agent in the treatment of cancer have all used intravenous injections of ATP in isolation. A proposed clinical trial of ATP for the treatment of melanoma would use ATP in conjunction with another anticancer agent. This is for 2 reasons. Firstly, the synergistic action of ATP with other anticancer agents has been demonstrated in *in vitro* studies of other cancer types (Maymon et al. 1994). Secondly, to obtain ethical approval for a clinical trial it is usual to design a trial with 2 arms, the first arm is established treatment and the second arm is established treatment plus new treatment. As there is some evidence for the use of chemotherapeutic agents in the treatment of melanoma a trial with one arm consisting of treatment with ATP and a second arm consisting of no treatment would be unlikely to gain regulatory approval.

Within the United Kingdom the two most widely used drugs in the treatment of advanced melanoma are interferon and dacarabzine (Bishop et al. 2002) and it would be possible to run a trial with one arm being treated with one of these two agents along with ATP. One further agent used in the treatment of melanoma is melphalan (Sarosy et al. 1988). Melphalan (L-phenylalanine mustard) is a bifunctional alkylating agent that is commonly administered orally to treat a wide variety of malignancies, including cancers of the breast and ovary, as well as multiple myeloma. The role of intravenous melphalan in cancer chemotherapy is not well

defined, despite its manageable toxicity and higher and more predictable blood levels following IV administration compared with oral administration. As a treatment for melanoma intravenous melphalan is not given systemically but by isolated limb perfusion (ILP) (Noorda et al. 2004). For non-resectable metastatic or recurrent melanoma in a limb (arm or leg) an alternative to conventional systemic chemotherapy is to use an alternative method of delivering the chemotherapy agent that maximizes tumour exposure. Isolated limb perfusion is a regional treatment technique that delivers high dose chemotherapy via a completely isolated vascular recirculating perfusion circuit as a means of regionally treating cancer. The major artery leading into the limb, and the major vein returning blood from the limb, have their blood supply diverted to a perfusion circuit with a similar design as a heart lung bypass machine (Fig 6.1).

The chemotherapy agent is injected into the limb circulation, but does not enter into the rest of the body's circulation. The limb's blood circulation is isolated from the rest of the body. After perfusing the limb for an hour with the high concentration chemotherapy agent, the limb is given a 'washout' and then reconnected normally to the systemic circulation. This allows higher concentration of toxic chemicals to be given to the limb, and spares the rest of the body the exposure to the agent and the side effects. The agent used for the treatment of melanoma with ILP is melphalan. By designing a trial for the treatment of patients with recurrent or metastatic limb melanoma with ILP with melphalan or melphalan with ATP the question can melanoma be treated with ATP can be answered. By using patients with

Figure 6.1



An isolated limb infusion (ILI) circuit.

Figure 6.1 modified from Linder et al 2002

recurrent or metastatic disease there is an available subpopulation of patients with a poor prognosis who would be able to enter the trial. The use of ILP allows a high dose of ATP to be given without systemic side effects and also allows monitoring of circulating ATP levels to confirm that a therapeutic dose is being delivered. Finally outcome can be measured by calculating tumour bulk in the diseased limb before and after the course of treatment by imaging techniques such as magnetic resonance imaging or positron emission topography.

Before this proposed trial can be commenced, an *in vitro* experiment needs to be performed where the effect of ATP in isolation, melphalan in isolation and ATP with melphalan on melanoma cell number is studied. If there is a synergistic effect by combining ATP with melphalan then this will be very strong evidence for a clinical trial of melphalan and ATP delivered by isolated limb perfusion.

There has been minimal previous work on purinergic signalling in malignant melanoma. This thesis has pioneered the investigation of the roles of purinergic receptors in melanoma and laid the foundations for further laboratory and clinical studies the therapeutic applications of modulating these receptors. The presence of functional P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2X<sub>7</sub> has been demonstrated at a molecular, cellular and tissue level. An agonist acting at the P2Y<sub>1</sub> and P2X<sub>7</sub> receptor subtypes and an antagonist acting at the P2Y<sub>2</sub> receptor subtype may be an effective treatment for melanoma. This may open a pathway for new treatment options for this important cancer.



## **References**

## Reference List

- Abbracchio MP, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA, Burnstock G (2003) Characterization of the UDP-glucose receptor (re-named here the P2Y<sub>14</sub> receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol Sci* 24:52-55
- Abbracchio MP, Burnstock G (1994) Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol Ther* 64:445-475
- Abbracchio MP, Burnstock G (1998) Purinergic signalling: pathophysiological roles. *Jpn J Pharmacol* 78:113-145
- Abraham EH, Okunieff P, Scala S, Vos P, Oosterveld MJ, Chen AY, Shrivastav B (1997) Cystic fibrosis transmembrane conductance regulator and adenosine triphosphate. *Science* 275:1324-1326
- Abraham EH, Salikhova AY, Rapaport E (2003) ATP in the Treatment of Advanced Cancer. In: Schwiebert EM (ed) *Extracellular Nucleotides and Nucleosides*. Elsevier Science, San Diego, pp. 415-452
- Abraham EH, Vos P, Kahn J, Grubman SA, Jefferson DM, Ding I, Okunieff P (1996) Cystic fibrosis hetero- and homozygosity is associated with inhibition of breast cancer growth. *Nat Med* 2:593-596
- Adrian K, Bernhard MK, Breitingner HG, Ogilvie A (2000) Expression of purinergic receptors (ionotropic P2X<sub>1-7</sub> and metabotropic P2Y<sub>1-11</sub>) during myeloid differentiation of HL60 cells. *Biochim Biophys Acta* 1492:127-138
- Agteresch HJ, Burgers SA, van der GA, Wilson JH, Dagnelie PC (2003a) Randomized clinical trial of adenosine 5'-triphosphate on tumor growth and survival in advanced lung cancer patients. *Anticancer Drugs* 14:639-644
- Agteresch HJ, Dagnelie PC, van der GA, Stijnen T, Wilson JH (2000) Randomized clinical trial of adenosine 5'-triphosphate in patients with advanced non-small-cell lung cancer. *J Natl Cancer Inst* 92:321-328
- Allman R, Cowburn P, Mason M (2000) In vitro and in vivo effects of a cyclic peptide with affinity for the  $\alpha(\text{nu})\beta 3$  integrin in human melanoma cells. *Eur J Cancer* 36:410-422
- Amizuka N, Warshawsky H, Henderson JE, Goltzman D, Karaplis AC (1994) Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. *J Cell Biol* 126:1611-1623
- Balch CM, Buzaid AC, Soong SJ, Atkins MB, Cascinelli N, Coit DG, Fleming ID, Gershenwald JE, Houghton A, Jr., Kirkwood JM, McMasters KM, Mihm MF, Morton DL, Reintgen DS, Ross MI, Sober A, Thompson JA, Thompson JF (2001) Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J Clin Oncol* 19:3635-3648

Balch CM, Soong SJ, Gershenwald JE, Thompson JF, Reintgen DS, Cascinelli N, Urist M, McMasters KM, Ross MI, Kirkwood JM, Atkins MB, Thompson JA, Coit DG, Byrd D, Desmond R, Zhang Y, Liu PY, Lyman GH, Morabito A (2001b) Prognostic factors analysis of 17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system. *J Clin Oncol* 19:3622-3634

Bari F, Ariwodola JO, Pleschka K (1993) Circulatory effects caused by intra-arterial infusion of AMP, ADP and ATP in the canine facial and nasal vascular beds. *J Vasc Res* 30:125-131

Bennett GC, Ford AP, Smith JA, Emmett CJ, Webb TE, Boarder MR (2003) P2Y receptor regulation of cultured rat cerebral cortical cells: calcium responses and mRNA expression in neurons and glia. *Br J Pharmacol* 139:279-288

Berridge MJ (1987) Inositol trisphosphate as a second messenger in signal transduction. *Ann N Y Acad Sci* 494:39-51

Berridge MJ (1995) Capacitative calcium entry. *Biochem J* 312 ( Pt 1):1-11

Bishop JA, Corrie PG, Evans J, Gore ME, Hall PN, Kirkham N, Roberts DL, Anstey AV, Barlow RJ, Cox NH (2002) UK guidelines for the management of cutaneous melanoma. *Br J Plast Surg* 55:46-54

Bodenham DC (1968) A study of 650 observed malignant melanomas in the South-West region. *Ann R Coll Surg Engl* 43:218-239

Bodin P, Burnstock G (2001a) Evidence that release of adenosine triphosphate from endothelial cells during increased shear stress is vesicular. *J Cardiovasc Pharmacol* 38:900-908

Bodin P, Burnstock G (2001b) Purinergic signalling: ATP release. *Neurochem Res* 26:959-969

Boissy RE (1998) Extracutaneous Melanocytes. In: Nordlund JJ, Boisse RE, Hearing VJ, King RA, Ortone JP (eds) *The Pigmentary System: Physiology and Pathophysiology*. Oxford University Press, New York, pp. 59-74

Born GV, Kratzer MA (1984) Source and concentration of extracellular adenosine triphosphate during haemostasis in rats, rabbits and man. *J Physiol* 354:419-429

Brackett LE, Daly JW (1994) Functional characterization of the A2b adenosine receptor in NIH 3T3 fibroblasts. *Biochem Pharmacol* 47:801-814

Breslow A (1970) Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. *Ann Surg* 172:902-908

Brocker EB, Magiera H, Herlyn M (1991) Nerve growth and expression of receptors for nerve growth factor in tumors of melanocyte origin. *J Invest Dermatol* 96:662-665

Brown JR, Cornell K, Cook PW (2000) Adenosine- and adenine-nucleotide-mediated inhibition of normal and transformed keratinocyte proliferation is dependent upon dipyrindamole-sensitive adenosine transport. *J Invest Dermatol* 115:849-859

- Burnstock G (1972) Purinergic nerves. *Pharmacol Rev* 24:509-581
- Burnstock G (1978) A basis for distinguishing two types of purinergic receptor. In: Straub RW, Bolis L (eds) *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach*. Raven Press, New York, pp. 107-118
- Burnstock G (1988) Sympathetic purinergic transmission in small blood vessels. *Trends Pharmacol Sci* 9:116-117
- Burnstock G (1990) Noradrenaline and ATP as cotransmitters in sympathetic nerves. *Neurochem Int* 17:357-368
- Burnstock G (1997) The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology* 36:1127-1139
- Burnstock G (1998) Sympathetic purinergic transmission in small blood vessels. *Trends in Pharmacological Science* 9:116-117
- Burnstock G (2000) P2X receptors in sensory neurones. *Br J Anaesth* 84:476-488
- Burnstock G (2001) Overview of P2 receptors: possible functions in immune cells 1174. *Drug Dev Res* 53:53-59
- Burnstock G (2002a) Potential therapeutic targets in the rapidly expanding field of purinergic signalling. *Clin Med* 2:45-53
- Burnstock G (2002b) Purinergic signaling and vascular cell proliferation and death. *Arterioscler Thromb Vasc Biol* 22:364-373
- Burnstock G (2003) Introduction: ATP and its metabolites as potent extracellular agonists. In: Schwiebert EM (ed) *Current Topics in Membranes, Vol 54. Purinergic Receptors and Signalling*. Academic Press, San Diego, pp. 1-27
- Burnstock G, Dumsday B, Smythe A (1972) Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. *Br J Pharmacol* 44:451-461
- Burnstock G, Kennedy C (1985) Is there a basis for distinguishing two types of P2-purinoceptor? *Gen Pharmacol* 16:433-440
- Burnstock G, Knight GE (2004) Cellular distribution and functions of P2 receptor subtypes in different systems. *Int Rev Cytol* 240:31-304
- Burnstock G, Williams M (2000) P2 purinergic receptors: modulation of cell function and therapeutic potential. *J Pharmacol Exp Ther* 295:862-869
- Burrell HE, Bowler WB, Gallagher JA, Sharpe GR (2003) Human keratinocytes express multiple P2Y-receptors: evidence for functional P2Y1, P2Y2, and P2Y4 receptors. *J Invest Dermatol* 120:440-447
- Calvert RC, Shabbir M, Thompson CS, Mikhailidis DP, Morgan RJ, Burnstock G (2004) Immunocytochemical and pharmacological characterisation of P2-

- purinoceptor-mediated cell growth and death in PC-3 hormone refractory prostate cancer cells. *Anticancer Res* 24:2853-2859
- Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN (1995) A P2X purinoceptor expressed by a subset of sensory neurons. *Nature* 377:428-431
- Chiozzi P, Sanz JM, Ferrari D, Falzoni S, Aleotti A, Buell GN, Collo G, Di Virgilio F (1997) Spontaneous cell fusion in macrophage cultures expressing high levels of the P2Z/P2X7 receptor. *J Cell Biol* 138:697-706
- Chow SC, Kass GE, Orrenius S (1997) Purines and their roles in apoptosis. *Neuropharmacology* 36:1149-1156
- Chueh SH, Kao LS (1993) Extracellular ATP stimulates calcium influx in neuroblastoma x glioma hybrid NG108-15 cells. *J Neurochem* 61:1782-1788
- Cook PW, Ashton NM, Pittelkow MR (1995) Adenosine and adenine nucleotides inhibit the autonomous and epidermal growth factor-mediated proliferation of cultured human keratinocytes. *J Invest Dermatol* 104:976-981
- Coutinho-Silva R, Persechini PM, Bisaggio RD, Perfettini JL, Neto AC, Kanellopoulos JM, Motta-Ly I, Dautry-Varsat A, Ojcius DM (1999) P2Z/P2X7 receptor-dependent apoptosis of dendritic cells. *Am J Physiol* 276:C1139-C1147
- Cowen DS, Berger M, Nuttle L, Dubyak GR (1991) Chronic treatment with P2-purinergic receptor agonists induces phenotypic modulation of the HL-60 and U937 human myelogenous leukemia cell lines. *J Leukoc Biol* 50:109-122
- Cressman VL, Lazarowski E, Homolya L, Boucher RC, Koller BH, Grubb BR (1999) Effect of loss of P2Y(2) receptor gene expression on nucleotide regulation of murine epithelial Cl(-) transport. *J Biol Chem* 274:26461-26468
- Czajkowski R, Lei L, Sabala P, Baranska J (2002) ADP-evoked phospholipase C stimulation and adenylyl cyclase inhibition in glioma C6 cells occur through two distinct nucleotide receptors, P2Y(1) and P2Y(12). *FEBS Lett* 513:179-183
- Denda M, Inoue K, Fuziwara S, Denda S (2002) P2X purinergic receptor antagonist accelerates skin barrier repair and prevents epidermal hyperplasia induced by skin barrier disruption. *J Invest Dermatol* 119:1034-1040
- Denton KJ, Stretch JR, Gatter KC, Harris AL (1992) A study of adhesion molecules as markers of progression in malignant melanoma. *J Pathol* 167:187-191
- Di Virgilio F, Borea PA, Illes P (2001) P2 receptors meet the immune system. *Trends Pharmacol Sci* 22:5-7
- Dixon CJ, Bowler WB, Littlewood-Evans A, Dillon JP, Bilbe G, Sharpe GR, Gallagher JA (1999) Regulation of epidermal homeostasis through P2Y2 receptors. *Br J Pharmacol* 127:1680-1686
- Dreiling L, Hoffman S, Robinson WA (1996) Melanoma: epidemiology, pathogenesis, and new modes of treatment. *Adv Intern Med* 41:553-604

- Drury AN, Szent-Györgyi A (1929) The physiological activity of adenine compounds with special reference to their action upon the mammalian heart  
14. J Physiol (Lond) 68:213-237
- Dubyak GR (2000) Purinergic signaling at immunological synapses. J Auton Nerv Syst 81:64-68
- Dubyak GR, De Young MB (1985) Intracellular Ca<sup>2+</sup> mobilization activated by extracellular ATP in Ehrlich ascites tumor cells. J Biol Chem 260:10653-10661
- Dunn PM, Zhong Y, Burnstock G (2001) P2X receptors in peripheral neurons. Prog Neurobiol 65:107-134
- Elder DE (1989) Human melanocytic neoplasms and their etiologic relationship with sunlight. J Invest Dermatol 92:297S-303S
- Estrela JM, Obrador E, Navarro J, Lasso De la Vega MC, Pellicer JA (1995) Elimination of Ehrlich tumours by ATP-induced growth inhibition, glutathione depletion and X-rays. Nat Med 1:84-88
- Ferrari D, La Sala A, Chiozzi P, Morelli A, Falzoni S, Girolomoni G, Idzko M, Dichmann S, Norgauer J, Di Virgilio F (2000) The P2 purinergic receptors of human dendritic cells: identification and coupling to cytokine release. FASEB J 14:2466-2476
- Fishman P, Bar-Yehuda S, Barer F, Madi L, Multani AS, Pathak S (2001) The A3 adenosine receptor as a new target for cancer therapy and chemoprotection. Exp Cell Res 269:230-236
- Fitzpatrick TB, Breathnach AS (1963) The epidermal melanin unit system. Dermatol Wochenschr 147:481-489
- Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 53:527-552
- Friedmann PS, Gilchrist BA (1987) Ultraviolet radiation directly induces pigment production by cultured human melanocytes. J Cell Physiol 133:88-94
- Froio J, Abraham EH, Soni R, Epstein A, Okunieff P (1995) Effect of intraperitoneal ATP on tumor growth and bone marrow radiation tolerance. Acta Oncol 34:419-422
- Gaba S, Didier C, Cohendy R, Prefaut C (1986) Effets vasculaires pulmonaires et systemique de l'ATP chez l'homme. C R Soc Biol 568-573
- Gargett CE, Wiley JS (1997) The isoquinoline derivative KN-62 a potent antagonist of the P2Z-receptor of human lymphocytes. Br J Pharmacol 120:1483-1490
- Gartland A, Hipskind RA, Gallagher JA, Bowler WB (2001) Expression of a P2X7 receptor by a subpopulation of human osteoblasts. J Bone Miner Res 16:846-856

- Gavrieli Y, Sherman Y, Ben Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119:493-501
- Gershwin ME, Ikeda RM, Kawakami TG, Owens RB (1977) Immunobiology of heterotransplanted human tumors in nude mice. *J Natl Cancer Inst* 58:1455-1461
- Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, Parks WP (1973) In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst* 51:1417-1423
- Gillespie JH (1934) The biological significance of the linkages in adenosine triphosphoric acid 3380. *J Physiol (Lond)* 80:345-359
- Gillies RJ, Didier N, Denton M (1986) Determination of cell number in monolayer cultures. *Anal Biochem* 159:109-113
- Gitay-Goren H, Halaban R, Neufeld G (1993) Human melanoma cells but not normal melanocytes express vascular endothelial growth factor receptors. *Biochem Biophys Res Commun* 190:702-708
- Glass R, Burnstock G (2001) Immunohistochemical identification of cells expressing ATP-gated cation channels (P2X receptors) in the adult rat thyroid. *J Anat* 198:569-579
- Going JJ, Gusterson BA (1999) Molecular pathology and future developments. *Eur J Cancer* 35:1895-1904
- Greig AV, James SE, McGrouther DA, Terenghi G, Burnstock G (2003a) Purinergic receptor expression in the regeneration epidermis in a rat model of normal and delayed wound healing. *Exp Dermatol* 12:860-871
- Greig AV, Linge C, Cambrey A, Burnstock G (2003b) Purinergic receptors are part of a signaling system for keratinocyte proliferation, differentiation, and apoptosis in human fetal epidermis. *J Invest Dermatol* 121:1145-1149
- Greig AV, Linge C, Healy V, Lim P, Clayton E, Rustin MH, McGrouther DA, Burnstock G (2003c) Expression of purinergic receptors in non-melanoma skin cancers and their functional roles in A431 cells. *J Invest Dermatol* 121:315-327
- Greig AV, Linge C, Terenghi G, McGrouther DA, Burnstock G (2003d) Purinergic receptors are part of a functional signaling system for proliferation and differentiation of human epidermal keratinocytes. *J Invest Dermatol* 120:1007-1015
- Grin-Jorgensen C.A, Rigel D.S., Friedman R.J (1992) The world-wide incidence of malignant melanoma. In: Balch C.M (ed) *Cutaneous Melanoma*. Lippencott, pp. 27-39
- Groschel-Stewart U, Bardini M, Robson T, Burnstock G (1999) Localisation of P2X5 and P2X7 receptors by immunohistochemistry in rat stratified squamous epithelia. *Cell Tissue Res* 296:599-605

- Grygorczyk R, Hanrahan JW (1997) CFTR-independent ATP release from epithelial cells triggered by mechanical stimuli. *Am J Physiol* 272:C1058-C1066
- Halaban R (1991) Growth factors regulating normal and malignant melanocytes. *Cancer Treat Res* 54:19-40
- Halaban R, Langdon R, Birchall N, Cuono C, Baird A, Scott G, Moellmann G, McGuire J (1988) Paracrine stimulation of melanocytes by keratinocytes through basic fibroblast growth factor. *Ann N Y Acad Sci* 548:180-190
- Hara M, Toyoda M, Yaar M, Bhawan J, Avila EM, Penner IR, Gilchrest BA (1996) Innervation of melanocytes in human skin. *J Exp Med* 184:1385-1395
- Haskell CM, Mendoza E, Pisters KM, Fossella FV, Figlin RA (1998) Phase II study of intravenous adenosine 5'-triphosphate in patients with previously untreated stage IIIB and stage IV non-small cell lung cancer. *Invest New Drugs* 16:81-85
- Haskell CM, Wong M, Williams A, Lee LY (1996) Phase I trial of extracellular adenosine 5'-triphosphate in patients with advanced cancer. *Med Pediatr Oncol* 27:165-173
- Hoebertz A, Arnett TR, Burnstock G (2003) Regulation of bone resorption and formation by purines and pyrimidines. *Trends Pharmacol Sci* 24:290-297
- Holroyde CP, Reichard GA, Jr. (1986) General metabolic abnormalities in cancer patients: anorexia and cachexia. *Surg Clin North Am* 66:947-956
- Hopfner M, Lemmer K, Jansen A, Hanski C, Riecken EO, Gavish M, Mann B, Buhr H, Glassmeier G, Scherubl H (1998) Expression of functional P2-purinergic receptors in primary cultures of human colorectal carcinoma cells. *Biochem Biophys Res Commun* 251:811-817
- Hoyle CHV, Burnstock G (1993) Postganglionic efferent transmission to the bladder and urethra. In: Maggi C (ed) *The Autonomic Nervous System, Vol. 3. Nervous Control of the Urogenital System*. Harwood Academic Publishers, Switzerland, pp. 349-383
- Hu DN, Woodward DF, McCormick SA (2000) Influence of autonomic neurotransmitters on human uveal melanocytes in vitro. *Exp Eye Res* 71:217-224
- Humphreys BD, Dubyak GR (1996) Induction of the P2z/P2X7 nucleotide receptor and associated phospholipase D activity by lipopolysaccharide and IFN-gamma in the human THP-1 monocytic cell line. *J Immunol* 157:5627-5637
- Humphreys BD, Rice J, Kertesz SB, Dubyak GR (2000) Stress-activated protein kinase/JNK activation and apoptotic induction by the macrophage P2X7 nucleotide receptor. *J Biol Chem* 275:26792-26798
- Inculet RI, Peacock JL, Gorschboth CM, Norton JA (1987) Gluconeogenesis in the tumor-influenced rat hepatocyte: importance of tumor burden, lactate, insulin, and glucagon. *J Natl Cancer Inst* 79:1039-1046



- Jacobson KA, Kim YC, King BF (2000) In search of selective P2 receptor ligands: interaction of dihydropyridine derivatives at recombinant rat P2X(2) receptors. *J Auton Nerv Syst* 81:152-157
- Janssens R, Boeynaems JM (2001) Effects of extracellular nucleotides and nucleosides on prostate carcinoma cells. *Br J Pharmacol* 132:536-546
- Kaczmarek E, Koziak K, Seigny J, Siegel JB, Anrather J, Beaudoin AR, Bach FH, Robson SC (1996) Identification and characterization of CD39/vascular ATP diphosphohydrolase. *J Biol Chem* 271:33116-33122
- Kanitakis J (1998) Immunohistochemistry of normal human skin. In: Kanitakis J, Vassileva S, Woodley D (eds) *Diagnostic Immunohistochemistry of the Skin*. Chapman & Hall, London, pp. 38-51
- Kanitakis J (2002) Anatomy, histology and immunohistochemistry of normal human skin. *Eur J Dermatol* 12:390-399
- Kasukawa H, Sugimoto M, Oshima N, Fujii R (1985) Control of chromatophore movements in dermal chromatic units of blue damselfish--I. The melanophore. *Comp Biochem Physiol C* 81:253-257
- Katzur AC, Koshimizu T, Tomic M, Schultze-Mosgau A, Ortmann O, Stojilkovic SS (1999) Expression and responsiveness of P2Y2 receptors in human endometrial cancer cell lines. *J Clin Endocrinol Metab* 84:4085-4091
- Kennedy C, Saville VL, Burnstock G (1986) The contributions of noradrenaline and ATP to the responses of the rabbit central ear artery to sympathetic nerve stimulation depend on the parameters of stimulation. *Eur J Pharmacol* 122:291-300
- Keppens S (1993) The complex interaction of ATP and UTP with isolated hepatocytes. How many receptors? *Gen Pharmacol* 24:283-289
- Khakh BS, Barnard EA, Burnstock G, Kennedy C, King BF, North RA, Séguéla P, Voigt M, Humphrey PPA (2000) P2X receptor-channels. In: Girdlestone D (ed) *The IUPHAR Compendium of Receptor Characterization and Classification*. IUPHAR Media Ltd., London, pp. 290-305
- King BF, Burnstock G, Boyer JL, Boeynaems J-M, Weisman GA, Kennedy C, Jacobson KA, Humphries RG, Abbracchio MP, Gachet C, Miras-Portugal MT (2000) The P2Y receptors. In: Girdlestone D (ed) *The IUPHAR Compendium of Receptor Characterization and Classification*. IUPHAR Media Ltd., London, pp. 306-320
- Kumazawa T, Fujii R (1984) Concurrent releases of norepinephrine and purines by potassium from adrenergic melanosome-aggregating nerve in Tilapia. *Comp Biochem Physiol C* 78:263-266
- Lasso de la Vega MC, Terradez P, Obrador E, Navarro J, Pellicer JA, Estrela JM (1994) Inhibition of cancer growth and selective glutathione depletion in Ehrlich tumour cells in vivo by extracellular ATP. *Biochem J* 298 ( Pt 1):99-105

- LERNER AB, SHIZUME K, FITZPATRICK TB, MASON HS (1954) MSH: the melanocyte-stimulating hormone. *AMA Arch Derm Syphilol* 70:669-674
- Lew RA, Sober AJ, Cook N, Marvell R, FITZPATRICK TB (1983) Sun exposure habits in patients with cutaneous melanoma: a case control study. *J Dermatol Surg Oncol* 9:981-986
- Li N, Mangini J, Bhawan J (2002) New prognostic factors of cutaneous melanoma: a review of the literature. *J Cutan Pathol* 29:324-340
- Libow LF, Scheide S, DeLeo VA (1988) Ultraviolet radiation acts as an independent mitogen for normal human melanocytes in culture. *Pigment Cell Res* 1:397-401
- Lin WW, Chuang DM (1993) Extracellular ATP stimulates inositol phospholipid turnover and calcium influx in C6 glioma cells. *Neurochem Res* 18:681-687
- Maaser K, Hopfner M, Kap H, Sutter AP, Barthel B, von Lampe B, Zeitz M, Scherubl H (2002) Extracellular nucleotides inhibit growth of human oesophageal cancer cells via P2Y(2)-receptors. *Br J Cancer* 86:636-644
- MacKenzie A, Wilson HL, Kiss-Toth E, Dower SK, North RA, Surprenant A (2001) Rapid secretion of interleukin-1beta by microvesicle shedding. *Immunity* 15:825-835
- Mangini J, Li N, Bhawan J (2002) Immunohistochemical markers of melanocytic lesions: a review of their diagnostic usefulness. *Am J Dermatopathol* 24:270-281
- Martin GN, Thom SA, Sever PS (1991) The effects of adenosine triphosphate (ATP) and related purines on human isolated subcutaneous and omental resistance arteries. *Br J Pharmacol* 102:645-650
- Maymon R, Bar-Shira MB, Cohen-Armon M, Holtzinger M, Leibovici J (1994) Enhancing effect of ATP on intracellular adriamycin penetration in human ovarian cancer cell lines. *Biochim Biophys Acta* 1201:173-178
- Mehta VB, Hart J, Wewers MD (2001) ATP-stimulated release of interleukin (IL)-1beta and IL-18 requires priming by lipopolysaccharide and is independent of caspase-1 cleavage. *J Biol Chem* 276:3820-3826
- Merighi S, Mirandola P, Milani D, Varani K, Gessi S, Klotz KN, Leung E, Baraldi PG, Borea PA (2002) Adenosine receptors as mediators of both cell proliferation and cell death of cultured human melanoma cells. *J Invest Dermatol* 119:923-933
- Merighi S, Varani K, Gessi S, Cattabriga E, Iannotta V, Ulouglu C, Leung E, Borea PA (2001) Pharmacological and biochemical characterization of adenosine receptors in the human malignant melanoma A375 cell line. *British Journal of Pharmacology* 134:1215-1226
- Miyashita Y, Kumazawa T, Fujii R (1984) Receptor mechanisms in fish chromatophores--VI. Adenosine receptors mediate pigment dispersion in guppy and catfish melanophores. *Comp Biochem Physiol C* 77:205-210

- Montesinos MC, Desai A, Chen JF, Yee H, Schwarzschild MA, Fink JS, Cronstein BN (2002) Adenosine promotes wound healing and mediates angiogenesis in response to tissue injury via occupancy of A(2A) receptors. *Am J Pathol* 160:2009-2018
- Montesinos MC, Gadangi P, Longaker M, Sung J, Levine J, Nilsen D, Reibman J, Li M, Jiang CK, Hirschhorn R, Recht PA, Ostad E, Levin RI, Cronstein BN (1997) Wound healing is accelerated by agonists of adenosine A2 (G alpha s-linked) receptors. *J Exp Med* 186:1615-1620
- Moore DJ, Chambers JK, Wahlin JP, Tan KB, Moore GB, Jenkins O, Emson PC, Murdock PR (2001) Expression pattern of human P2Y receptor subtypes: a quantitative reverse transcription-polymerase chain reaction study. *Biochim Biophys Acta* 1521:107-119
- Morrone FB, Jacques-Silva MC, Horn AP, Bernardi A, Schwartzmann G, Rodnigh R, Lenz G (2003) Extracellular nucleotides and nucleosides induce proliferation and increase nucleoside transport in human glioma cell lines. *J Neurooncol* 64:211-218
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55-63
- Murakami S, Hashikawa T, Saho T, Takedachi M, Nozaki T, Shimabukuro Y, Okada H (2001) Adenosine regulates the IL-1 beta-induced cellular functions of human gingival fibroblasts. *Int Immunol* 13:1533-1540
- Mure T, Sano K, Kitagawa T (1992) Modulation of membrane permeability, cell proliferation and cytotoxicity of antitumor agents by external ATP in mouse tumor cells. *Jpn J Cancer Res* 83:121-126
- Murphy G (1997) Histology of the skin. In: Elenitsas R, Jaworsky C, Johnson B (eds) *Lever's Histopathology of the Skin*. Lippincott-Raven, Philadelphia, pp. 5-50
- Murphy GF, Lopansri S, Mihm MC, Jr. (1985) Capsule dermatopathology: clinicopathologic types of malignant melanoma--relevance to biologic behavior and diagnostic surgical approach. *J Dermatol Surg Oncol* 11:674-682
- Nakamura E, Uezono Y, Narusawa K, Shibuya I, Oishi Y, Tanaka M, Yanagihara N, Nakamura T, Izumi F (2000) ATP activates DNA synthesis by acting on P2X receptors in human osteoblast-like MG-63 cells. *Am J Physiol Cell Physiol* 279:C510-C519
- Namoto S (1987) A subtype of adenosine receptors mediating pigment dispersion in leucophores of the medaka: evidence for an A2-receptor. *Comp Biochem Physiol C* 88:75-81
- Nayak KK, Maity P, Bhattacharyya R, Chatterjee M (1990) Antitumour activities of copper-ATP complex on transplantable murine lymphoma. *Pharmacology* 41:350-356
- Neary JT (2000) Trophic actions of extracellular ATP: gene expression profiling by DNA array analysis. *J Auton Nerv Syst* 81:200-204

- Neary JT, Rathbone MP, Cattabeni F, Abbracchio MP, Burnstock G (1996) Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. *Trends Neurosci* 19:13-18
- Nicholson DW, Thornberry NA (1997) Caspases: killer proteases. *Trends Biochem Sci* 22:299-306
- Nicke A, Baumert HG, Rettinger J, Eichele A, Lambrecht G, Mutschler E, Schmalzing G (1998) P2X1 and P2X3 receptors form stable trimers: a novel structural motif of ligand-gated ion channels. *EMBO J* 17:3016-3028
- Noorda EM, Vrouenraets BC, Nieweg OE, Van Coevorden F, Kroon BB (2004) Isolated limb perfusion: what is the evidence for its use? *Ann Surg Oncol* 11:837-845
- Norris DA, Horikawa T, Morelli JG (1994) Melanocyte destruction and repopulation in vitiligo. *Pigment Cell Res* 7:193-203
- Nylund G, Nordgren S, Delbro DS (2004) Expression of P2Y2 purinoceptors in MCG 101 murine sarcoma cells, and HT-29 human colon carcinoma cells. *Auton Neurosci* 112:69-79
- Obrador E, Navarro J, Mompo J, Asensi M, Pellicer JA, Estrela JM (1997) Glutathione and the rate of cellular proliferation determine tumour cell sensitivity to tumour necrosis factor in vivo. *Biochem J* 325 ( Pt 1):183-189
- Oglesby IB, Lachnit WG, Burnstock G, Ford AP (1999) Subunit specificity of polyclonal antisera to the carboxy terminal regions of P2X receptors, P2X1 through P2X7. *Drug development and research* 47:189-195
- Osborne JE (2002) Skin cancer screening and surveillance. *Br J Dermatol* 146:745-754
- Per LK, Jon HA, Dissing S (2002) The human SH-SY5Y neuroblastoma cell-line expresses a functional P2X7 purinoceptor that modulates voltage-dependent Ca<sup>2+</sup> channel function. *J Neurochem* 83:285-298
- Popper LD, Batra S (1993) Calcium mobilization and cell proliferation activated by extracellular ATP in human ovarian tumour cells. *Cell Calcium* 14:209-218
- Quinn M, Babb P, Brock A, Kirby L, Jones J. Cancer trends in England and Wales 1950-1999. 2004. National Statistics Office.  
Ref Type: Report
- Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol Rev* 50:413-492
- Rapaport E (1983) Treatment of human tumor cells with ADP or ATP yields arrest of growth in the S phase of the cell cycle. *J Cell Physiol* 114:279-283
- Rapaport E (1988) Experimental cancer therapy in mice by adenine nucleotides. *Eur J Cancer Clin Oncol* 24:1491-1497

- Rapaport E (1990) Mechanisms of anticancer activities of adenine nucleotides in tumor-bearing hosts. *Ann N Y Acad Sci* 603:142-149
- Rapaport E, Fishman RF, Gercel C (1983) Growth inhibition of human tumor cells in soft-agar cultures by treatment with low levels of adenosine 5'-triphosphate. *Cancer Res* 43:4402-4406
- Rapaport E, Fontaine J (1989a) Anticancer activities of adenine nucleotides in mice are mediated through expansion of erythrocyte ATP pools. *Proc Natl Acad Sci U S A* 86:1662-1666
- Rapaport E, Fontaine J (1989b) Generation of extracellular ATP in blood and its mediated inhibition of host weight loss in tumor-bearing mice. *Biochem Pharmacol* 38:4261-4266
- Roman RM, Fitz JG (1999) Emerging roles of purinergic signaling in gastrointestinal epithelial secretion and hepatobiliary function. *Gastroenterology* 116:964-979
- Ryten M, Dunn PM, Neary JT, Burnstock G (2002) ATP regulates the differentiation of mammalian skeletal muscle by activation of a P2X5 receptor on satellite cells. *J Cell Biol* 158:345-355
- Sarosy G, Leyland-Jones B, Soochan P, Cheson BD (1988) The systemic administration of intravenous melphalan. *J Clin Oncol* 6:1768-1782
- Sauer H, Hescheler J, Wartenberg M (2000) Mechanical strain-induced  $\text{Ca}^{2+}$  waves are propagated via ATP release and purinergic receptor activation. *Am J Physiol Cell Physiol* 279:C295-C307
- Schafer R, Sedehizade F, Welte T, Reiser G (2003) ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells. *Am J Physiol Lung Cell Mol Physiol* 285:L376-L385
- Schultze-Mosgau A, Katzur AC, Arora KK, Stojilkovic SS, Diedrich K, Ortmann O (2000) Characterization of calcium-mobilizing, purinergic P2Y(2) receptors in human ovarian cancer cells. *Mol Hum Reprod* 6:435-442
- Schwiebert LM, Rice WC, Kudlow BA, Taylor AL, Schwiebert EM (2002) Extracellular ATP signaling and P2X nucleotide receptors in monolayers of primary human vascular endothelial cells. *Am J Physiol Cell Physiol* 282:C289-C301
- Sellers LA, Simon J, Lundahl TS, Cousens DJ, Humphrey PP, Barnard EA (2001) Adenosine nucleotides acting at the human P2Y1 receptor stimulate mitogen-activated protein kinases and induce apoptosis. *J Biol Chem* 276:16379-16390
- Serrone L, Hersey P (1999) The chemoresistance of human malignant melanoma: an update. *Melanoma Res* 9:51-58
- Slater M, Danieleto S, Pooley M, Cheng TL, Gidley-Baird A, Barden JA (2004) Differentiation between cancerous and normal hyperplastic lobules in breast lesions. *Breast Cancer Res Treat* 83:1-10

- Slater M, Scolyer RA, Gidley-Baird A, Thompson JF, Barden JA (2003a) Increased expression of apoptotic markers in melanoma. *Melanoma Res* 13:137-145
- Slater MD, Lauer C, Gidley-Baird A, Barden JA (2003b) Markers for the development of early prostate cancer. *J Pathol* 199:368-377
- Smolle J, Soyer HP, Kerl H (1989) Proliferative activity of cutaneous melanocytic tumors defined by Ki-67 monoclonal antibody. A quantitative immunohistochemical study. *Am J Dermatopathol* 11:301-307
- Sneddon P, Burnstock G (1984) Inhibition of excitatory junction potentials in guinea-pig vas deferens by alpha, beta-methylene-ATP: further evidence for ATP and noradrenaline as cotransmitters. *Eur J Pharmacol* 100:85-90
- Solini A, Chiozzi P, Falzoni S, Morelli A, Fellin R, Di Virgilio F (2000) High glucose modulates P2X7 receptor-mediated function in human primary fibroblasts. *Diabetologia* 43:1248-1256
- Solini A, Chiozzi P, Morelli A, Fellin R, Di Virgilio F (1999) Human primary fibroblasts in vitro express a purinergic P2X7 receptor coupled to ion fluxes, microvesicle formation and IL-6 release. *J Cell Sci* 112 ( Pt 3):297-305
- Solle M, Labasi J, Perregaux DG, Stam E, Petrushova N, Koller BH, Griffiths RJ, Gabel CA (2001) Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem* 276:125-132
- Soong SJ, Shaw HM, Balch CM, McCarthy WH, Urist MM, Lee JY (1992) Predicting survival and recurrence in localized melanoma: a multivariate approach. *World J Surg* 16:191-195
- Stein TP (1978) Cachexia, gluconeogenesis and progressive weight loss in cancer patients. *J Theor Biol* 73:51-59
- Stoop R, Thomas S, Rassendren F, Kawashima E, Buell G, Surprenant A, North RA (1999) Contribution of individual subunits to the multimeric P2X(2) receptor: estimates based on methanethiosulfonate block at T336C. *Mol Pharmacol* 56:973-981
- Straume O, Akslen LA (1997) Alterations and prognostic significance of p16 and p53 protein expression in subgroups of cutaneous melanoma. *Int J Cancer* 74:535-539
- Surprenant A, Rassendren F, Kawashima E, North RA, Buell G (1996) The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). *Science* 272:735-738
- Torres GE, Egan TM, Voigt MM (1999) Hetero-oligomeric assembly of P2X receptor subunits. Specificities exist with regard to possible partners. *J Biol Chem* 274:6653-6659
- Tulapurkar ME, Laubinger W, Nahum V, Fischer B, Reiser G (2004) Subtype specific internalization of P2Y1 and P2Y2 receptors induced by novel adenosine 5'-O-(1-boranotriphosphate) derivatives. *British Journal of Pharmacology* 142:869-878

- Ujhazy P, Berleth ES, Pietkiewicz JM, Kitano H, Skaar JR, Ehrke MJ, Mihich E (1996) Evidence for the involvement of ecto-5'-nucleotidase (CD73) in drug resistance. *Int J Cancer* 68:493-500
- UKCCCR (1998) United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (Second Edition). *Br J Cancer* 77:1-10
- Unwin RJ, Bailey MA, Burnstock G (2003) Purinergic signaling along the renal tubule: the current state of play. *News Physiol Sci* 18:237-241
- Wang Q, Wang L, Feng YH, Li X, Zeng R, Gorodeski GI (2004) P2X7 receptor-mediated apoptosis of human cervical epithelial cells. *Am J Physiol Cell Physiol* 287:C1349-C1358
- Wang Y, Roman R, Lidofsky SD, Fitz JG (1996) Autocrine signaling through ATP release represents a novel mechanism for cell volume regulation. *Proc Natl Acad Sci U S A* 93:12020-12025
- Wegner CD (2001) Novel mechanistic targets for the treatment of sub-acute and chronic bronchitis. *Curr Pharm Des* 7:199-212
- White N, Butler PEM, Burnstock G (2005a) Human melanomas express functional P2X7 receptors. *Cell Tissue Research* 321(3): 411-418
- White N, Ryten M, Clayton E, Butler P, Burnstock G (2005b) P2Y purinergic receptors regulate the growth of human melanomas. *Cancer Lett* 224:81-91
- Wiley JS, Gargett CE, Zhang W, Snook MB, Jamieson GP (1998) Partial agonists and antagonists reveal a second permeability state of human lymphocyte P2Z/P2X7 channel. *Am J Physiol* 275:C1224-C1231
- Woodhouse EC, Amanatullah DF, Schetz JA, Liotta LA, Stracke ML, Clair T (1998) Adenosine receptor mediates motility in human melanoma cells. *Biochem Biophys Res Commun* 246:888-894
- Yaar M, Eller MS, DiBenedetto P, Reenstra WR, Zhai S, McQuaid T, Archambault M, Gilchrist BA (1994) The trk family of receptors mediates nerve growth factor and neurotrophin-3 effects in melanocytes. *J Clin Invest* 94:1550-1562
- Yegutkin GG, Henttinen T, Jalkanen S (2001) Extracellular ATP formation on vascular endothelial cells is mediated by ecto-nucleotide kinase activities via phosphotransfer reactions. *FASEB J* 15:251-260
- Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK (2001) Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N Engl J Med* 345:494-502
- Zhang XJ, Zheng GG, Ma XT, Yang YH, Li G, Rao Q, Nie K, Wu KF (2004) Expression of P2X7 in human hematopoietic cell lines and leukemia patients. *Leuk Res* 28:1313-1322

Zimmermann H (2001) Ectonucleotidases: Some Recent Developments and a Note on Nomenclature. *Drug Dev Res* 52:44-56

Di Virgilio F, Chiozzi P, Ferrari D, Falzoni S, Sanz JM, Morelli A, Torboli M, Bolognesi G, Baricordi OR (2001) Nucleotide receptors: an emerging family of regulatory molecules in blood cells. *Blood* 97(3):587-600.

Linder P, Doubrovsky A, Kam PC, Thompson JF (2002) Prognostic factors after isolated limb infusion with cytotoxic agents for melanoma. *Ann Surg Oncol* 9(2):127-36.

MacKie. Malignant melanoma: a guide to early diagnosis. 1994.



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Page 24

Figure modified from The Pigmentary System; Norlund 1998.

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(Bishop et al. 2002) *replaced with* (MacKie et al. 1994)

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Lentigo melanoma melanoma *replaced with* Lentigo maligna melanoma

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Figures modified from Burnstock 2006

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Figure 1.10B modified from Di Virgilio et al 2001

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Isolated limb perfusion *replaced by* Isolated limb infusion

Figure 6.1 modified from Linder et al 2002

Complete reference (White et al 2005a) cited

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Three additional references:

Di Virgilio F, Chiozzi P, Ferrari D, Falzoni S, Sanz JM, Morelli A, Torboli M, Bolognesi G, Baricordi OR (2001) Nucleotide receptors: an emerging family of regulatory molecules in blood cells. *Blood* 97(3):587-600.

Linder P, Doubrovsky A, Kam PC, Thompson JF (2002) Prognostic factors after isolated limb infusion with cytotoxic agents for melanoma.

*Ann Surg Oncol* 9(2):127-36.

MacKie. *Malignant melanoma: a guide to early diagnosis*. 1994.